Seed functional traits in crops and their wild relatives

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Author's declaration

I declare that I am the sole author of the work presented in this thesis and that it is original unless otherwise indicated. This thesis has been supervised by Dr. Charlotte Seal and Prof. Hugh Pritchard at the Royal Botanic Gardens, Kew (UK) and Prof. Bill Finch-Savage at the University of Warwick. The research was funded by a European Union funded project (EcoSeed, Impacts of Environmental Conditions on Seed Quality).

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Abstract

Seed functional traits, such as seed mass, germination, seedling production and longevity are of paramount importance to agriculture, food security and the conservation of wild species. However, species adaptability is usually described by plant functional traits and seed functional traits are underrepresented in ecological studies. Thus, this thesis has the purpose to compare seed functional traits of crops and their crop wild relatives (CWRs) especially seed germination rate through thermal and hydro time models and normal seedling survival. Moreover, the influence of the maternal and progeny environment on seed trait responses was also explored.

Seed functional traits (physical, physiological) were characterised for three crops (*Hordeum vulgare, Brassica oleracea,* and *Helianthus annuus*), produced under control and climate-change scenarios (water limitations), and a wide range of their CWRs.

Crop seeds had relatively poor conversion of germinated seeds into normal seedlings (lower than 30 %) at 35 ºC or higher temperatures and -1.0 MPa compared with CWR seeds. Thermal and hydro time parameters differed greatly between the crops and their CWRs. These functional traits were influenced by the environment of seed collection site including mean monthly precipitation for *Brassica* CWRs and annual mean temperature for *Hordeum* and *Helianthus* CWRs. Thus, the maternal environment contributed to the seed functional trait response. Not only are thermal- and hydro-time and their thresholds for temperature and water potential reliable descriptors of seed lot performance under broad ranges of temperature and water potential, these parameters also effectively describe the loss of germination quality in sunflower seeds during ageing.

In conclusion, this comparison of the underlying variability and factors influencing the measured parameters of seed responses in crops and their CWRs emphasises the importance of preserving and using CWR genetic resources in future crop breeding programmes.

List of Abbreviations

Abbreviation	Meaning
ANOVA	Analysis of variance
CV	Coefficient of variation
CWRs	Crop wild relatives
ºC	Degrees Celsius
DF	Degrees of Freedom
EcoSeed	Impacts of Environmental Conditions on Seed Quality
eRH	Equilibrium relative humidity
FAO	Food and Agriculture Organisation of the United Nations
G	Germination
g	Grams
GA	Gibberellic acid
GR	Germination rate
h	Hour
IPCC	Intergovernmental Panel on Climate Change
IPK	Leibniz Institute of Plant Genetics and Crop Plant Research
ISTA	International Seed Testing Association
Ki	Initial viability of a seed lot
LiCl	Lithium Chloride
m.a.s.l	Metres above sea level
MC	Moisture content
M	Molar
mg	milligram
mM	Millimolar
mm	millimetre
MoG	Month of germination
MPa	MegaPascal
MSB	Millennium Seed Bank
NInf	Non-infected
NMR	Nuclear magnetic resonance
NoSC	Non-scarified
Norm	Normal irrigation
p50	Time in days for viability to drop to 50%
PEG	Polyethylene glycol
PNS%	Normal seedlings in proportion of germinated seeds
RH	Relative humidity
S	Normal seedlings
SC	Scarified
SD	Standard deviation
SID	Kew Seed Information Database
Stop	Stopped irrigation

t	Time
T	Temperature
t20	Time to reach 20 % of germination
t50	Time to reach 50 % of germination
t80	Time to reach 80 % of germination
T_b	Base temperature
T_c	Ceiling temperature
T_o	Optimal temperature
TTC	2,3,5-Tripheniltetrazolium chloride
v	Viability
K_E	Moisture viability constant is the potential longevity
C_W	Moisture viability constant as humidity coefficient
C_H	Temperature viability constant
C_Q	Quadratic temperature viability constant
θ_H	Hydro time
θ_{HT}	Hydrothermal time
θ_T	Thermal time of the sub-optimal range of temperatures
θ_{Tsupra}	Thermal time of the supra-optimal range of temperatures
Ψ	Water potential
Ψ_b	Base water potential

1 CHAPTER 1: INTRODUCTION

1.1 Crops and crop wild relatives

There are more than 300,000 plant species across the world (Christenhusz & Byng, 2016). In 1990 the available data showed that 90 % of the main food for human consumption (cereals and legumes represent more than 60 %) was supplied by just 103 plant species (Prescott-Allen & Prescott-Allen, 1990). However, at least 2500 plant species can be considered as crops (Dirzo & Raven, 2003).

Over the next 50 years drought and elevated temperature resulting from climate change are predicted to have a major impact in Europe and North America (Walck *et al.*, 2011; IPCC, 2013). In particular, warming temperatures and fluctuations in precipitation are known to increase the risk of yield loss in the most economically important crops, such as wheat, rice, maize and barley (Porter & Semenov, 2005; Lobell & Field, 2007; Newton *et al.*, 2011a). Germination and seedling establishment are key traits in the determination of yield and these early traits are likely to be greatly impacted by climate change (Dornbos & Mullen, 1991). Furthermore, crops have been bred with predictable and uniform germination in conditions of low stress and this may result in an inability to adapt to future variations of environmental conditions (Gepts, 2010). Therefore, climate change may exacerbate economic losses in agriculture by reductions in crop yield resulting from an impact on germination and seedling establishment.

The survival of crop wild relatives (CWRs, wild species with a close genetic relationship to a crop), under future climate scenarios may be more likely because of their greater genetic variability and their adaptability due to more plasticity in their traits compared to crops. Some reports have revealed that plasticity of seed traits in this early plant stage may facilitate seedling establishment to a greater range of environmental conditions (Cochrane *et al.*, 2015a). However, it is not known whether CWRs have greater resilience to climate change than their crop relatives, during germination and normal seedling growth and this question forms the central theme to this thesis.

Seeds of crops and CWRs of three genera, *Hordeum, Brassica* and *Helianthus* are studied in this thesis. They belong to the Poaceae, Brassicaceae and Asteraceae families respectively that represent the three largest families of crop production (cereals, vegetables and oil seeds). These families contain important crops such as *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus* respectively that are included in the Annex 1 of the International Treaty on Plant Genetic Resources for Food and Agriculture (FAO, 2009). The global agricultural production of the three crops was more than 250 million tonnes in 2014 (FAOSTAT, 2017), which is approximately 2.5 % of the crop global production.

1.1.1 Plant genera selected

The *Hordeum* genus belongs to the Poaceae family of more than 10,000 species of which cereals are the most economically important crop species. The crop barley (*H. vulgare*) originated in the Fertile Crescent of the Middle East (Harlan, 1968; Nevo, 1992). Today, barley is distributed over the world from 330 m below sea level (close to the Dead Sea) up to 4200 m in the Andes (Bolivia) (Akar *et al.,* 2004). Barley is used for both intensive production and subsistence farming. It is important economically for both animal and human consumption (Newman & Newman, 2006), and the global production in 2016 stood at 145 million tonnes (FAOSTAT, 2017). Barley was one of the earliest crops to be domesticated, and this resulted in greater productivity (higher yielding) and larger grains (seeds) than its CWRs (Badr *et al.,* 2000). Wild *Hordeum* species are widespread in both hemispheres from tropical (South America) to arctic (Central Asia) regions (von Bothmer et al., 1995). *Hordeum* CWRs are annual or perennial, occupying habitats in central and west North America (for example, *H. pusillum*) and the Mediterranean area (for example, *H. bulbosum*, *H. murinum* and *H. marinum*). In these areas, drought and high temperatures are predominant environmental conditions (von Bothmer *et al.,* 1995).

The *Brassica* genus belongs to the Brassicaceae family of more than 3700 species. The family is distributed worldwide (Anjum *et al.,* 2012) over the Mediterranean region, south Asia, Britain and France (Tsunoda *et al.,* 1980) and thus can exist in a wide range of environmental conditions. *Brassica* is

economically the most important genus of the family because it includes edible roots crops, vegetables and oilseeds (Tsunoda *et al.,* 1980). The global production of *Brassica* crops was more than 70 million tonnes in 2016 (FAOSTAT, 2017). The genus possesses a wide morphological and genetic diversity (Arias et al., 2014). In addition, *Brassica* CWRs have interesting physiological adaptations such as the ability to accumulate metal ions from contaminated soils or drought and salt tolerance (Tsunoda *et al.,* 1980; Fahey *et al.,* 2001; Ozturk *et al.,* 2008; Kumar *et al.,* 2012).

The *Helianthus* genus belongs to the Asteraceae family, one of the largest families of more than 30,000 species. The genus *Helianthus* consists of 49 species. From the classification of Timme et al. (2007) on *Helianthus* species, 37 are perennial (e.g., *H. angustifolius* and *H. pumilus*) and only 12 are annual species, including *H. annuus* and *H. argophyllus*. The genus is well-known for the crop *H. annuus* (sunflower), which is one of the most lucrative oilseeds in the world. The centre of origin and first domestication area for *H. annuus* was North America (Blackman *et al.,* 2011). Nowadays, sunflower is cultivated extensively and contributes towards the economy in all continents except Antarctica (Seiler & Gulya, 2015) and the global production was 47 million tonnes of seeds in 2016 (FAOSTAT, 2017). The genus possesses an enormous diversity of species (Kane *et al.,* 2013), reflecting their adaptation to many habitats. Most of the *Helianthus* CWRs possess tolerance to biotic and abiotic stress. For example, annual CWRs have resistance to sunflower rust (fungus); *H. glaucophyllus* is a potential source of *Phomopsis* brown stem (fungus) resistance and *H. argophyllus* has been used in breeding for drought tolerance (Seiler *et al.,* 2017).

1.1.2 Domestication: advantages and disadvantages

Domestication is the result of a selection process over years that lead to adaptation of plants to cultivation and utilisation by humans (Gepts, 2010). The discovery of useful plants (crops) and their domestication has been dated back to 8000 BC in wheat and barley (Zohary & Hopf, 2000). Since then, farmers have continued with domestication by selecting desirable plant and seed traits. Originally traits were selected on the basis of phenotypic responses such as greater seed size, the absence of or weak seed dormancy, plant growth

homogeneity, higher yield and the loss of natural seed dispersal (Zohary & Hopf, 2000; Fuller, 2007; Brown *et al.*, 2009; Gepts, 2010; Preece *et al.*, 2017). In the twenty-first century, cross-pollination between crops and local wild relatives (i.e., CWRs) was explored to improve crops. For example, the use of *Triticum turgidum* for its tolerance to salt (James *et al.*, 2006) in breeding programs of wheat and the CWR *Hordeum vulgare* subsp. *spontaneum* to introduce alleles that are resistant to mildew and rust to barley (Schmalenbach *et al.*, 2008). Nowadays, sophisticated processes are utilised to improve crops using biotechnology and genetic knowledge (Wolfe, 2011).

One of the most common and well-known consequences of crop domestication and selection is the reduction of genetic variation denominated as "genetic bottleneck" (Eyre-Walker *et al.*, 1998). The selection of a few individuals in a population causes reduced genetic diversity, and as a consequence the populations can be vulnerable to biotic and abiotic factors such as diseases or environmental changes (Hancock, 2012). Some beneficial traits obtained from wild species (i.e., drought tolerance or pests resistance) may be associated with other non-desirable traits or traits that may have detrimental effect (i.e., seed dormancy or reduction in yield) (Milla *et al.*, 2015; Migicovsky & Myles, 2017). According to FAO (1998) the genetic diversity of crops has been reduced by 75 % compared to the wild relatives. Cereals are reported as the most affected crops suffering genetic erosion (defined as the loss of genes or alleles as well as varieties) due to overexploitation, population pressures, changing agricultural systems and pests and diseases (FAO, 2010). Possible consequences of genetic erosion are the increase of vulnerability of the populations to pests or lack of adaptation to environmental changes.

1.1.3 Importance of conserving CWRs

Wild species possess an enormous variability of plant functional traits that contribute towards ecosystem services (Díaz *et al.*, 2013). Natural selection has left high genetic diversity across the wild species of the world, which has been exploited since the first half of the 20th century in agricultural programs of crop breeding (Meilleur & Hodgkin, 2004; Hajjar & Hodgkin, 2007). Because of their use in crop breeding programmes as sources of adaptive traits to environmental stresses, understanding the natural variation in CWRs is

increasingly important (Dempewolf *et al.,* 2014). CWRs are wild species with a close genetic relationhip to a crop, and thus are an important source of trait variation for improving agriculture in the future. Generally, the concept of CWRs is used in agriculture, however it is appropiate to other plants such as, medicinal, ornamentals or forestry species (Maxted *et al.*, 2006). CWRs can provide beneficial traits to crops, such as resilience to pests and diseases or tolerance to abiotic stressess (Maxted, 2003; Maxted *et al.*, 2011; Dempewolf *et al.*, 2014). Nowadays, most of the main crops used to feed the world population have alleles from their CWRs (Maxted & Kell, 2009), in fact, CWRs are an important component of the Plant Genetic Resources for Food and Agriculture (PGRFA) (FAO, 2010).

Some CWRs can be overexploited and then they are more vulnerable due to genetic erosion (i.e., loss of genetic diversity) (Maxted, 2003; Bettencourt *et al.*, 2008) or their habitats might be destroyed. For example, in Georgia and South Caucasus, wild grapevine is disappearing because of deforestation (Akhalkatsi *et al.*, 2012). Other CWRs can suffer from genetic pollution (gene flow from crops or foreign species to CWRs populations) that may have detrimental effects in addition to the loss of diversity (Maxted & Guarino, 2006; Bettencourt *et al.*, 2008). For example, the genetic diversity of wild populations of ryegrass in UK have been reduced by their crop (Sackville Hamilton, 1999). Therefore, the preservation of CWRs is of paramount importance. In particular, the CWRs related to important crops that contribute to the global economy and food security, are of high priority. The preservation of CWRs has been developed *in situ* and *ex situ*. Nowadays, 7.4 million *ex situ* accessions of plant genetic resources for food and agriculture (PGRFA) are available worldwide. However, CWRs are generally underrepresented in *ex situ* conservation programs (FAO, 2010; Castañeda-Álvarez *et al.*, 2016). From a global and detailed study of more than 1000 taxa of CWRs related to 81 crops, it was found that more than 50 % of taxa were under-represented or missed in *ex situ* conservation gene banks (Castañeda-Álvarez *et al.,* 2016). Moreover, above 70 % of taxa are of high priority for conservation, such as the CWRs of fruits (banana and mango), forages (alfalfa), sugar crops (sugarcane), starchy roots (cassava) and vegetables (eggplant). In the Millennium Seed Bank of the Royal Botanic Gardens, Kew (UK) there are already a diverse collection of seeds of over 37,000 wild plant

species from across the world, from which over 300 are CWRs. Currently, a CWRs project managed by the Global Crop Diversity Trust (Crop Trust) and the Royal Botanic Gardens, Kew, promotes the conservation of important CWRs prioritising the ones that are not represented in *ex situ* gene banks (https://www.cwrdiversity.org/). On the other hand, *in situ* conservation has been simultaneously managed to complement *ex situ* conservation, to maintain genetic variation and evolution of plant traits of CWRs (Meilleur & Hodgkin, 2004; Maxted & Kell, 2009). Both conservation strategies have pros and cons depending on complexity, time, space, effectiveness and economical resources (Maxted *et al.*, 2013).

In general, the populations of CWRs are better adapted to natural environments, and they have larger genetic diversity than crops, some of them possess plastic traits that allow them to survive under several environments (Nicotra *et al.*, 2010). A plastic trait can be defined as the expression of a genotype that varies in response to the environment (Bradshaw, 1965). The identification and characterisation of plastic traits in CWRs is highly relevant for future plant breeding. For example, wild species of *Hordeum* and *Brassica* have shown tolerance to drought (Nevo & Chen, 2010; Salisbury & Barbetti, 2011), and wild species of *Helianthus* possess resilience to biotic stresses (Seiler *et al.*, 2017).

As Milla *et al.* (2015) suggested, a greater understanding of the changes that crops have gone through during domestication requires extensive research. In their review, they focused on seed mass as a component of yield and not on other important functional seeds traits such as germination rate or thresholds. However, they declare that the effect of selection resulting in increased seed size might have other unknown consequences. The main aim of my project was to conduct a comparative study of seed functional traits between CWRs and crops including morphological, physiological and phenotypical seed traits.

1.2 Seed functional traits

Quantitative traits that regulate individual fitness through physiological, morphological, developmental or phenotypical mechanisms are defined as functional traits (Geber & Griffen, 2003). Global plant variability can be described by six plant functional traits, only one of which is a reproductive trait

(seed mass) rather than vegetative (Díaz *et al.*, 2016). These can be used in comparative biology to characterise the ecological responses of plants (Ackerly *et al.*, 2000; Grimes, 2001; Garnier & Navas, 2012). In recent years, investigations of seed functional traits in relation to the environment have been conducted (Cochrane *et al.*, 2011; Cochrane *et al.*, 2015a; Walck *et al.*, 2011; Seal *et al.*, 2017). Nonetheless, seed functional traits are still under- represented in plant ecology studies (Jiménez-Alfaro *et al.*, 2016), apart from morphological seed traits such as seed mass (Cornelissen *et al.*, 2003; Perez-Camacho *et al.*, 2012; Díaz *et al.*, 2013; Milla *et al.*, 2015). However, other seed functional traits of a physiological nature are of paramount importance to agriculture, food security and the conservation of wild species. These are described below.

1.2.1 Seed morphology

Seed mass is an important physical trait widely studied in relation to the environment or to the fitness of a seed population (Cornelissen *et al.*, 2003; Moles & Westoby, 2003; Moles *et al.*, 2007; Perez-Camacho *et al.*, 2012; Díaz *et al.*, 2013). In agriculture, seed mass and seed size have been linked to the development of larger plants from which the production of higher yields such as grain is expected (Preece *et al.*, 2017). In ecology, seed mass is a trait that is known to be responsive to environmental fluctuations (Roach & Wulff, 1987; Donohue *et al.*, 2005a; Moles *et al.*, 2007; Pakeman *et al.*, 2008; Nicotra *et al.*, 2010). In particular, the environment impacts on seed mass during the seed filling stage. For example, positive correlations were found between seed mass and annual rainfall (Harel *et al.*, 2011) and with mean annual temperature (Murray *et al.*, 2004). Seed mass may also be associated with other traits such as seed germination rate (Norden *et al.*, 2009), however the trend is not consistent among species. For example, Leishman *et al.* (2000) and Moles and Westoby (2004a) found that seeds with greater mass germinated faster, but Grime *et al.* (1981) and Kikuzawa and Koyama (1999) found the opposite. Therefore, the relationship between seed mass and seed germination, in addition to other physiological seed functional traits, is explored in subsequent chapters.

A seed develops from a mature female gametophyte (ovule) fertilised by a sperm cell of a male gametophyte (pollen). In angiosperms, three principal seed components, the embryo, endosperm (fusion of both male and female

gametophytes) and the seed coat and testa (develops from the ovule integuments) constitute a mature seed (Bewley *et al.*, 2013). The pericarp is the outer layer of the achenes composed of dead cells and is formed from the ovary wall. Mature seeds possess different types of embryos. A mature embryo contains a hypocotyl (stem), radicle (root) and one or two cotyledons when the seeds are monocotyledons or dicotyledons, respectively (Bewley *et al.*, 2013). All seeds possess endosperm, but only in endospermic seeds is the endosperm fully developed as a storage tissue providing reserves to the embryo during the germination process. In non-endospermic seeds, the cotyledons are the storage tissue. The shape and length of the embryo and endosperm and their relation to other structures varies among species.

One outer structure that surrounds the embryo, cotyledons and endosperm is the seed coat. The seed coat varies in structure according to the specific features of the ovule (female gametophyte), such as number and thickness of integuments, the pattern of vascularization, and the developmental changes in the integuments during seed maturation (Esau, 1977). For this reason, the structure and anatomy of the seed coat has been used taxonomically to differentiate between genera and species (Koul *et al.*, 2000; Zeng *et al.*, 2004; Yang *et al.*, 2012). Additionally, the seed coat is a protective structure: in some cases, it prevents water and oxygen penetrating the embryo (i.e. types of physical dormancy, section 1.2.2).

These morphological traits are not often characterised in CWRs and hence few comparisons have been made between CWRs and their crops. In Chapter 3, detailed information of seed morphological traits of CWRs and comparisons with crop seed lots are provided. Furthermore, relationships between these traits of CWRs and the environment of seed collection site are studied (Chapter 3).

1.2.2 Seed dormancy

Dormancy is defined as the temporary failure of the seed to germinate even under conditions considered “optimal” (in terms of water availability and temperature). Primary dormancy is the condition applied to seeds at shedding from the mother plant. This prevents early germination while maturing on the mother plant (i.e. preharvest sprouting in cereals) (Paulsen & Auld, 2004) or germination during the wrong time of the year where the environmental

conditions might be precarious to the seedling (Bewley, 1997). Seed dormancy can originate in the embryo or in the seed coat (Baskin & Baskin, 1998; Murdoch & Ellis, 2000). Once seeds lose primary dormancy, or even if they were previously non-dormant, they may acquire secondary dormancy due to subsequent stressful conditions in the soil. For example, anaerobic conditions or prolonged periods of high temperatures (Murdoch & Ellis, 2000; Bewley *et al.*, 2013). The release of both primary and secondary dormancy is governed by environmental cues, such as temperature, light, nitrate or smoke components (Bewley *et al.*, 2013). There are four main classes of dormancy (Baskin & Baskin, 1998; Fenner & Thompson, 2004):

- Morphological: the embryo is undeveloped or immature when shed and a period of time is required for embryo development to be completed before germination.

- Physiological: the most frequent form of dormancy (Finch-Savage & Leubner-Metzger, 2006). Germination is repressed until a physiological change occurs in the seed. This type of dormancy can be deep or non-deep (Baskin & Baskin, 2004). Non-deep physiological dormancy can be released with hormones (e.g., gibberellins, GA), cold or warm stratification or by dry after-ripening (i.e., the seeds lose dormancy after being exposed to dry conditions for a period of time) (Murdoch & Ellis, 2000). Deep physiological dormancy requires a combination of GA and seed scarification and /or longer periods of stratification to terminate seed dormancy (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006).

- Morphophysiological: the seed has immature and undeveloped embryos but also requires physiological changes to germinate.

- Physical: the seed possesses an impermeable barrier to water (seed coat, testa, integuments or pericarp) and it is necessary to break down or scarify the barrier for germination to begin.

Seed dormancy is an un-desirable trait for crops and has been selected against during domestication so that only very short periods of after-ripening are required. Nonetheless, physiological dormancy may be present after seed harvest in some cereals and in sunflower seeds (Corbineau *et al.*, 1990; Benech-Arnold & Sánchez, 2004). In cereals, seed dormancy is necessary to avoid precocious germination before harvest (e.g., pre-harvest sprouting in barley).

However, long-lasting dormancy is a problem for the seed industry. The timing and duration of dormancy depends on the physiology and the genotype of the plant, but also the maternal environment (Benech-Arnold & Sánchez, 2004). For example, lower temperatures during seed development can induce deeper and longer periods of seed dormancy (Fenner, 1991).

Many CWRs possess dormancy that under natural conditions, ensure seeds only germinate during the appropriate season and when optimal conditions are available for seedling survival. However, the presence of dormancy in crops means seedling emergence is less predictable (Bewley *et al*., 2013; Baskin & Baskin, 1998; Finch-Savage & Leubner-Metzger, 2006). Understanding the range of conditions in which CWRs germinate, as well as their capacity to convert into normal seedling across a range of temperatures and water availabilities is critical to breeding programmes. The lack of knowledge and characterisation of seed germination traits, such as seed dormancy, limits the use of CWRs in breeding programmes.

1.2.3 Seed germination and normal seedlings

Seed germination is the process where the embryo develops and grows to emerge from the seed. The process begins with water uptake by the seed (imbibition) and ends with the emergence of the embryonic axis, usually the radicle, through the structures surrounding it, followed by the hypocotyl and the cotyledon(s) (Bewley, 1997). Thus, seed germination is usually determined as radicle emergence.

Seed germination is most often controlled by temperature and water availability (Heydecker, 1977; Bewley *et al.*, 2013). It can be considered as a plastic trait that responds to environmental fluctuations (Roach & Wulff, 1987; Donohue *et al.*, 2005a; Nicotra *et al.*, 2010). Plastic traits are important as the survival of species under environmental change will be based on their capacity to adapt to new environments and survival will depend on how quickly a species responds to climate change (Nicotra *et al.*, 2010).

Following the definition of ISTA (2017), normal seedlings show the potential for continued development into satisfactory plants. For that, normal seedlings must have all their essential structures well developed and healthy, e.g., green hypocotyls and cotyledons and long and white roots. A lack of light, water or

nutrients (competition from other seedlings or plants) are the major causes of death in seedlings in the field. Other factors can be important including, predation, diseases or pathogens and physical factors such as soil crusting (Fenner & Thompson, 2004; Bewley *et al.*, 2013). Moreover, environmental parameters such as high temperature or drought (Wellington & Noble, 1985; Sacchi & Price, 1992; Laman, 1995) can result in seedling death.

Seed germination and their conversion into normal seedlings are two important seed functional traits that are thought to represent the most sensitive plant stages to environmental changes (Lloret *et al.*, 2004; Fay & Schultz, 2009; Dalgleish *et al.*, 2010; Kimball *et al.*, 2010). Thus, in this thesis both functional traits are explored in crops and CWRs under several temperature and water conditions.

1.2.4 Seed longevity

Seed longevity is the capacity of a seed lot to maintain viability over time with the survival time dependent on the specific storage condition, whether in the soil seed bank or during *ex situ* storage. It is widely known that the main parameters that can be controlled to increase the life-span or longevity of the seeds preserved *ex situ* are storage temperature and humidity. As a general rule, seed longevity doubles with a 1 % decrease in seed moisture content (MC) or 5 °C decrease in the storage temperature for orthodox seeds (Harrington, 1972).

The storage behaviour of seeds with improved longevity on drying and cooling is called orthodox (Roberts, 1973). Most of the world's seed-bearing plants (92 %) are predicted to produce seeds that are orthodox (Wyse & Dickie, 2017). Seed longevity is usually assessed by ageing seeds under controlled environmental conditions, followed by germination testing. Besides the loss of viability, the process of ageing or deterioration of a seed before its death, results in a decrease of seed performance such as germination rate, total germination and/or the production of normal seedlings (i.e., indication of loss of vigour, described below) (Ellis & Roberts, 1981; Priestley, 1986). This loss of vigour is extremely important for the seed industry since it may contribute to economic losses (Finch-Savage & Bassel, 2015). For example, unexpected environmental changes (non-optimal conditions) at sowing time can negatively affect seedling

establishment in the field when using lower quality seed lots, resulting in a reduction in the scheduled planting density of crops to obtain the highest yield.

Understanding how long seeds can be stored and ways to improve seed storage is important for the maintenance and development of long-term *ex situ* seed banks. In addition, it is also advantageous for seed companies or farmers that store seeds for shorter periods of time, i.e. one year.

1.2.5 Seed vigour

Seed vigour has been widely described as a complex trait and thus its definition includes several properties. The International Seed Testing Association defined seed vigour as '*the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments*' (ISTA, 2017). Germination rate is an important and widely used descriptor of vigour (Pollock & Roos, 1972). In agriculture, a seed is considered vigorous when it is not dormant, has rapid germination and a healthy seedling emerges. Agriculture and food industry seek to maximise yield and emergence uniformity at optimal conditions and these are the key traits selected in crops, however seedling survival in adverse conditions has not been improved yet (Finch-Savage & Bassel, 2015). On the other hand, in CWRs, great diversity, heterogeneity and ability to germinate under stressful conditions may be an indication of seed vigour. This may provide an adaptive strategy of CWRs to survive across a wider range of environmental conditions in addition to successfully deal with unexpected changes in the environment.

The interactions between genotype and environmental factors are enormous and can determine seed vigour (Pollock & Roos, 1972; Finch-Savage & Bassel, 2015). Thus, seed vigour is difficult to define, however the quantification of several seed functional traits may contribute to a better description of the potential seed performance in the three genera studied.

1.3 Impact of the environment on seed functional traits

Early developmental stages (i.e., seed germination and seedling establishment) of plants are thought to be more sensitive to environmental fluctuations than adult stages (Lloret *et al.*, 2004; Fay & Schultz, 2009; Dalgleish *et al.*, 2010; Kimball *et al.*, 2010). How the maternal environment

impacts on seed functional traits is not fully understood (Forcella *et al.*, 2000; Walck *et al.*, 2011). Precipitation and temperature are the main environmental factors experienced by the mother plant that have an impact on subsequent seed functional traits such as seed mass, germination rate or seedling establishment (Dornbos & Mullen, 1991; Baskin & Baskin, 1998; Ackerly *et al.*, 2000; Peñuelas *et al.*, 2004; Porter, 2005; Menzel *et al.*, 2006; Franks *et al.*, 2007). In addition to the maternal environment, the progeny environment (i.e., the germination environment), also has implications for seed functional traits such as seed germination, dormancy release and seedling emergence (Alexander & Wulff, 1985; Dornbos, 1995; Donohue *et al.*, 2010).

Seed functional traits, especially seed germination and normal seedling growth are now recognised as critical components of the multiple environmentally regulated factors that define the ecological niche for population growth (Grubb, 1977; Poschlod *et al.*, 2013; Dürr *et al.*, 2015; Fernández-Pascual *et al.*, 2015). As a consequence of fluctuations of climatic conditions, seed dormancy status may be altered (Steadman *et al.*, 2004) or the timing of seed germination may change (Cochrane *et al.*, 2015b). Therefore, a comprehensive study and characterisation of the seed functional traits of CWRs in relation to the maternal environment and the progeny environment is necessary to understand the impact of environmental change.

The plants that suffer water stress tend to delay their growth processes such as flowering or seed filling, due to water deficit (Blum, 1996). These delays can affect the production of the plant causing yield losses. Moreover, drought can reduce the growth and size of plant organs and hence final yield will also be affected. However, the responses of plant traits to drought are variable and depend on the environmental conditions and the species. For example, in contrast to the trend of delaying processes, early flowering was found to be the strategy of *Brassica rapa* plants to avoid drought periods in natural conditions (Franks *et al.*, 2007) and in controlled conditions (Franks, 2011).

In addition to drought tolerance, salt tolerance has been explored in crops and CWRs. Drought and salinity can reduce seed germination rate and total germination when the seeds are subjected to presence of salt or limiting water in the seedbed. The tolerance of the species can be explored in the field under natural conditions by studying arid deserts or salt marshes (Kane & Rieseberg,

2007), by modifying the maternal environment by limiting irrigation (Meyer & Allen, 1999; Farahani *et al.*, 2010) or under controlled conditions simulating water stress using polyethylene glycol (Bradford, 1990; Dell'Aquila, 1992; Rowse & Finch-Savage, 2003; Soltani *et al.*, 2017), and/or salt (Kabar & Bartepe, 1990; Mer *et al.*, 2000; Farahani *et al.*, 2010). In this thesis the approach used in the crop genotypes was to limit irrigation during the seed filling stage. Additionally, polyethylene glycol (PEG) was used to simulate drought during seed germination under controlled conditions in both, crop and CWR seeds.

Many approaches can be used to quantify the effect of temperature on plant life cycle (Körner & Hiltbrunner, 2018) (Figure 1.1). For example, the "absolute minimum or maximum" temperature refers to the survival of an extreme event (e.g., damage of buds or flower due to extreme temperature of one night). On the other hand, the influence of mean temperature may have an effect over months, such as on metabolism or during the seed filling stage (Körner & Hiltbrunner, 2018). In this thesis, for geo-referenced CWR seed lots I consider both longer-term measurements of the environment, through the historical annual mean temperature and mean monthly precipitation (Figure 1.1), as well as the mean temperature and precipitation of the estimated month of germination (see Chapter 2, section 2.2.2). This is in contrast to the crop seed lots where controlled environmental conditions related to temperature and withholding of water were applied to the mother plant at specific development stages.

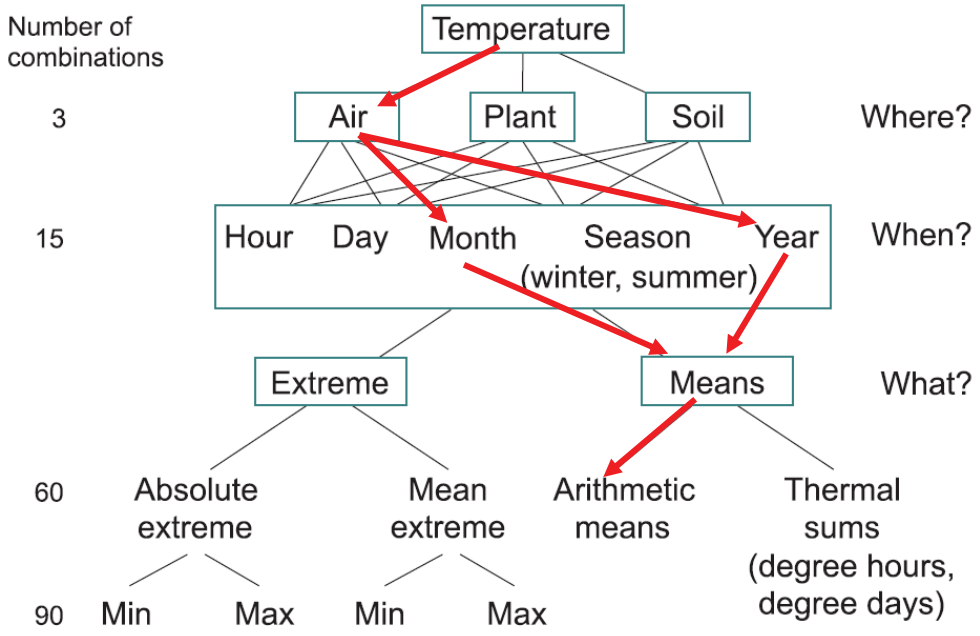


Figure 1.1 Possible combinations of the effect of temperature in the plant growth environment published in Körner & Hiltbrunner (2017), Figure 1. The red arrows indicate the chosen route for describing the environmental temperature of the CWRs studied in this thesis (annual mean temperature and mean temperature of the predicted month of germination).

1.4 Uses and relevance of thermal, hydro and hydrothermal time models

Seed germination responses to environmental temperature, water potential, or a combination of both, can be encapsulated in population-based models called thermal time (Bierhuizen & Wagenvoort, 1974; García-Huidobro *et al.*, 1982; Ellis *et al.*, 1986; Moot *et al.*, 2000; Trudgill *et al.*, 2000), hydro time (Covell *et al.*, 1986; Kebreab & Murdoch, 1999b; Kebreab & Murdoch, 2000; Finch-Savage & Leubner-Metzger, 2006; Windauer *et al.*, 2012) and hydrothermal time (Gummerson, 1986; Dahal & Bradford, 1994; Bradford, 1995; Allen *et al.*, 2000; Köchy & Tierlbörger, 2007) respectively. As these models are described in detail in Chapter 2, these will only be briefly described here.

Temperature is the main environmental factor affecting seed germination when water is not limited (Probert, 2000; Fenner & Thompson, 2004). The germination response to accumulated temperature can be modelled using the thermal time approach (Covell et al., 1986, Ellis et al., 1987, Pritchard and Manger, 1990, Bradford, 1995, Hardegree, 2006). In the thermal time (θ_T)

model seeds accumulate heat units (°Ch) to germinate in a given thermal time. When seeds are germinated at a range of temperatures the germination rate (GR, reciprocal of time) describes a positive (sub-optimal range of temperatures) or negative regression line (supra-optimal range of temperatures) when plotted against mean temperatures in most species. The intercept of the two lines is the estimated optimal temperature (T_o) where the GR is maximum. Base temperature (T_b) and ceiling temperature (T_c) are the intercept of the line when GR is predicted to be zero, where below T_b and above T_c germination cannot take place (García-Huidobro *et al.*, 1982).

The ability of the seeds to germinate in different water potentials can be calculated using hydro time (θ_H) (Gummerson, 1986). Seeds require hydro time units (MPah) to germinate in a given hydro time. A positive regression line is defined between GR and the water potential. The base water potential (Ψ_b) is calculated as the intercept of the regression line when the GR is zero.

The hydrothermal time (θ_{HT}) model describes germination time of a seed population across temperatures and water potentials (Gummerson, 1986), generally in the sub-optimal range of temperatures (between T_b and T_o). This model can quantify the interaction and effect of temperature and water potential conditions. θ_{HT} has been widely used as a predictor of dormancy loss (Christensen *et al.*, 1996; Bauer *et al.*, 1998; Meyer *et al.*, 2000; Bradford, 2002; Bair *et al.*, 2006; Finch-Savage & Leubner-Metzger, 2006) and as a descriptor of the capacity for seed germination under a range of environmental conditions. It is possible to identify the potential traits to deal with changes in the environment by analysing the performance of seeds under a wide range of conditions (Milla *et al.*, 2015).

These modelling approaches describe the response of the whole seed population and as such are a powerful means to analyse the germination response to environmental change. Nevertheless, very little has been reported about the characterisation of germination thresholds (T_b, T_c and Ψ_b) in relation to the maternal environment. Orrù *et al.* (2012) reported a correlation between thermal time for seed germination and the altitude of alpine species. Furthermore, Seal *et al.* (2017) found the mean temperature of the wettest quarter of the year as the best environmental predictor of germination in the sub-optimal temperature range (i.e., T_b to T_o) in 32 cactus species. In this thesis all

three population-based models are studied in the selected crop and CWR species to compare responses and to assess any relationship between the model parameters and the seed collection environment.

1.4.1 Can thermal, hydro and hydrothermal time models be applied to germination in the field?

The majority of studies using the thermal time model for characterisation of seed germination under different temperature conditions are laboratory or glasshouse based. However, this does not exclude the applicability of the thermal time model to predicting germination in the field (Finch-Savage & Phelps, 1993; Finch-Savage *et al.*, 1998; Allen *et al.*, 2000; Bradford, 2002). Some studies have found discrepancies in the seedling emergence time between the field environment and under controlled conditions (Finch-Savage & Phelps, 1993; Finch-Savage *et al.*, 1998) especially when water is scarce, and the soil temperature varies with depth soil and hence sowing depth. However, it seems that generally, the germination thresholds are reliable descriptors to estimate the temperature and water potential limits of a seed lot in field environments (Finch-Savage, 2004). A modification of the hydrothermal time model, which includes an interaction between temperature and water potential, could predict seedling emergence with more accuracy by using the germination thresholds established for a seed lot and knowing the soil water potential and temperature (Benech Arnold *et al.*, 1990; Forcella *et al.*, 2000; Roman *et al.*, 2000). For example, Kebreab & Murdoch (1999b) and Eizenberg *et al.* (2012) use a modification of the θ_T to estimate the emergence of a parasitic weed for future control on crops. Porceddu *et al.* (2013) estimated θ_T values under controlled conditions allowed prediction of the time of emergence in the field by *in situ* observations in an endemic tree species in Mediterranean climates. This study considered the effects of emergence from open and shaded areas with variation in temperatures. In summary, evidence exists that the models used in this thesis can be reliable descriptors of seed germination performance in the field and are relevant to predict the impact of future climate change scenarios.

1.5 Selected species

This study was aligned with the EcoSeed (Impacts of Environmental Conditions on Seed Quality) project; an EU funded project formed by 11 partners including University of Warwick (UK), Royal Botanic Gardens, Kew (UK), Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, IPK (Germany), Université Pierre et Marie Curie, UPMC (France) and Limagrain Europe (France). EcoSeed sought to obtain new knowledge of how stress impacts on seed quality in three crops, *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus* and their respective CWRs. These three crops are included in the Annex 1 of the International Treaty on Plant Genetic Resources for Food and Agriculture (FAO, 2009). The crop seeds were provided by the partners: *Hordeum vulgare* seeds by IPK, *Brassica oleracea* seeds by University of Warwick, and *Helianthus annuus* seeds by the seed company Limagrain. These crops were grown under limiting water conditions during the seed filling stage (detailed in Chapter 2, section 2.2.3). Additionally, seeds of each crop were bought from a commercial seed company, B&T World Seed (France). All the CWRs were supplied by the Royal Botanic Gardens, Millennium Seed Bank, Kew (the complete list of seed lots is detailed in Chapter 2 section 2.2). Database georeferenced information was used to select CWRs across environmental gradients based on the historical mean monthly precipitation and annual mean temperature obtained from GPS coordinates. The species were also selected according to the availability of seeds and the permission from the donor country to work with the material. CWRs species from two different environments where chosen when possible (detailed list of CWRs in Chapter 2, Table 2.3).

1.6 Aims

The vast majority of seed characterisation research has been performed on crop species. In contrast, little is known about the potential impact of the environment (temperature and water) on seed functional traits in CWRs. To address these limitations, a comparative study of seed functional traits is presented in this thesis by characterising CWRs and comparison with their crops. The importance of the diversity of traits to cope with the environmental changes of the CWRs and the possibility to use desirable traits to improve crops is discussed in the subsequent chapters. Furthermore, the influence of the

maternal environment on crops, such as water treatments during seed filling, and the impact of the environment of seed collection site (temperature and precipitation) on CWRs is analysed.

The following aims contribute towards the overall objective on my PhD:

- To characterise seed functional traits of wild seed lots of different species of *Hordeum, Brassica* and *Helianthus* and compare them to these traits in their crop relatives.
- To use population-based threshold models to estimate the thresholds for seed germination under controlled conditions of temperature and water potentials for crops and their wild relatives.
- To compare the conversion of germinated seeds into normal seedlings of CWRs and crops under a wide range of conditions.
- To characterise seed functional traits during artificial seed ageing in *Helianthus annuus*.
- To identify seed functional traits that contribute towards vigour and seed performance.

The analyses carried out can be used to assess the relevance of seed functional traits in CWRs for the improvement of crop seed performance in a future with predicted climate change.

1.7 Organisation of the thesis

The thesis structure of the experimental chapters is described in Figure 1.2 to encapsulate the general aim of this PhD.

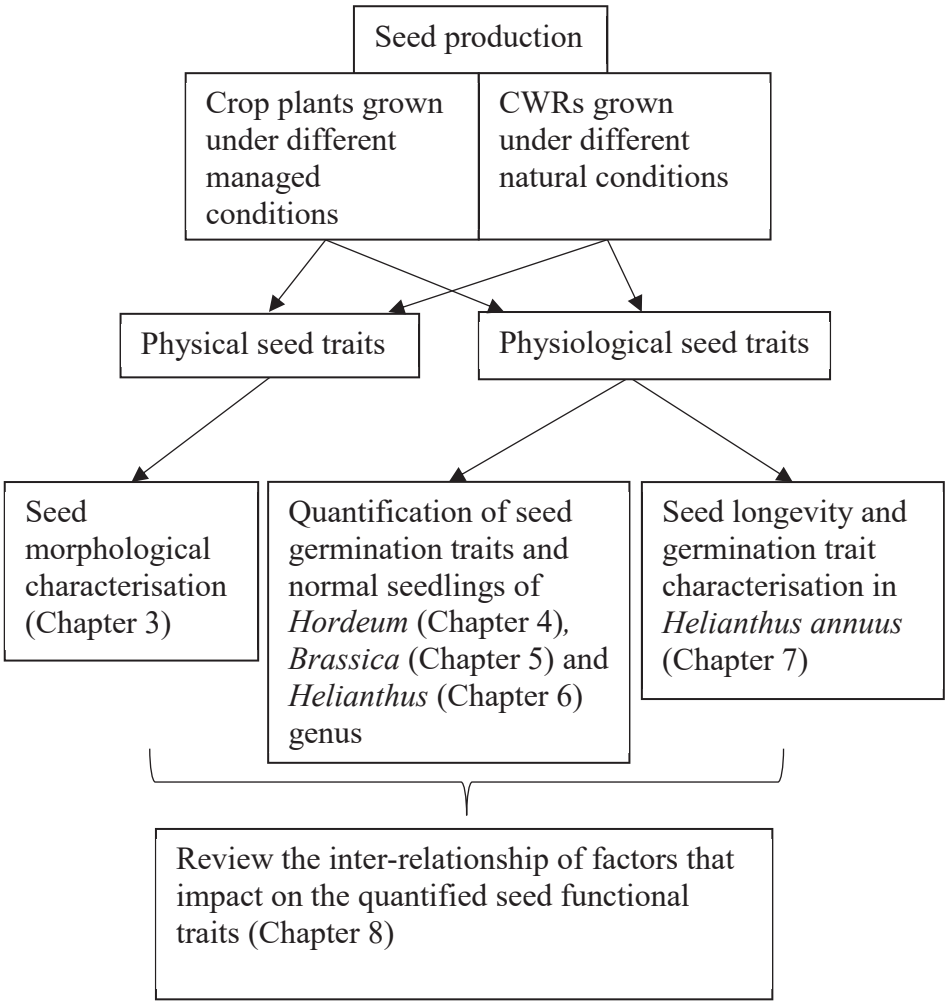


Figure 1.2 Scheme of the structure of the thesis from the experimental chapters to the general discussion.

2 CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals

2.1.1 Polyethylene Glycol

Polyethylene Glycol (PEG) 8000 (BP233.1 Fisher Scientific, Loughborough, UK) was used to create solutions with different water potential. PEG is an osmotic agent without a toxic effect on seeds and with sufficient molecular weight to prevent entry into the seeds (Michel, 1983; Khajeh-Hosseini *et al.*, 2003; Mehra *et al.*, 2003; Kaya *et al.*, 2006). PEG was dissolved in distilled water at different concentrations.

There is an interaction between water potential and temperature, therefore solutions of the same water potential were prepared at each temperature (Table 2.1) using the Equation 2.1 (Michel, 1983; Hardegree & Emmerich, 1990):

$$\Psi = (0.130\ PEG^2)*T - 13.7\ PEG^2 \qquad \text{Equation 2.1}$$

where Ψ is the water potential in megapascals (MPa), PEG is the grams of PEG/ g H_2O and T is the temperature (°C) at which the solution will be used. To confirm the accuracy of Equation 2.1, the MPa of the solutions made for use at 20 °C were measured at that temperature using an osmometer (osmometer automatic, Camlab, UK).

Table 2.1 Polyethylene glycol concentrations. Weight (g) of polyethylene glycol (PEG) per grams of water (g of PEG / g H_2O) needed to obtain the water potential established at each temperature (T) based on Equation 2.1.

T (°C)	-0.3 MPa	-0.5 MPa	-0.8 MPa	-1.0 MPa
15	0.1598	0.2063	0.2609	0.2917
20	0.1644	0.2122	0.2685	0.3002
25	0.1694	0.2187	0.2767	0.3093

2.1.2 Gibberellic acid

Gibberellic acid (GA_3) was used to break dormancy in *Helianthus* CWRs seeds. GA_3 (G7645 Sigma Aldrich, Gillingham, UK) was diluted in one litre of phosphate citrate buffer, that was prepared by mixing 1.7 mM citric acid (anhydrous, 99 %; 10020760, Fisher Scientific, UK) and 3.3 mM potassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$) (11367688, Fisher Scientific, UK) adjusted to pH 5 with 1 M NaOH (Bentsink *et al.*, 2006). However, for the

results to be comparable to other germination experiments in non-dormant species (germinated in water), it was tested whether germination using a 5 mM GA_3 solution at pH 5 gave the same results as 5 mM GA_3 solution at pH 7 adjusted with 1 M NaOH (Chapter 6). There was no significant difference ($P > 0.05$) between the seeds germinated at pH 5 and pH 7. Thus, it was decided to prepare 0.5 mM, 1.0 mM, 2.5 mM, 5 mM, 7.5 mM and 10 mM GA_3 at pH 7 for all dormancy breaking treatments to select the most effective concentration. All GA_3 solutions were stored at 5 °C until use.

2.1.3 Lithium chloride

Air environments with different relative humidities (RH; 30 %, 45 %, 60 % and 75 %) were created using solutions of lithium chloride (LiCl) to artificially age sunflower seeds under controlled conditions. Between 20 to 50 g of LiCl (≥ 99 %, 10491241, Fisher Scientific, UK) were added slowly to one litre of distilled water (Table 2.2). The LiCl solution was poured in sealed boxes (electrical enclosure boxes, 300 x 300 x 130 mm, Ensto Ltd, Southampton, UK) and placed at 20 °C, 30 °C and 40 °C ± 2 °C (section 2.8). To ensure that the RH did not change over time, 10 mL of the solution were placed into hygrometers (AW-D10 water activity probe, HygroPalm, Rotronic instruments Ltd, Crawley, UK) to measure the RH of the air in equilibrium with the solution every three to four weeks. The RH did not change and thus did not require adjustment.

Table 2.2 Relative humidities (RH) percentages obtained above solutions of the salt lithium chloride (LiCl). The RH measurements obtained from the hygrometer had a standard error of ± 2 %.

RH %	grams LiCl per 100 mL H_2O
30	52
45	39
60	30
75	22.5

2.1.4 Tetrazolium solution

2,3,5-triphenyltetrazolium chloride (tetrazolium chloride or TTC, ≥95%, T8877, Sigma Aldrich, UK) was used to test the viability of non-germinated

seeds by staining its living tissues. One gram of TTC (1 %) was dissolved in 100 mL of phosphate buffer. Buffer solution were prepared with distilled water in two separate solutions. The first solution was 9.078 g of potassium dihydrogen orthophosphate (KH_2PO_4) (11400113, Fisher Scientific, UK) dissolved in 1000 mL of water. For the second solution, 11.876 g of disodium hydrogen orthophosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) (10666652, Fisher Scientific, UK) was dissolved in 1000 mL of water. Then, two parts of the first solution were mixed with three parts of the second solution. The solution was adjusted to pH 7 using 1 M sodium hydroxide (NaOH) if needed (ISTA, 1985). The solution was stored at 4 °C in a glass bottle that excluded the light for up to three months.

2.2 Seed material

2.2.1 Crop wild relatives (CWRs)

All the seed accessions held in the Millennium Seed Bank, Royal Botanic Gardens, Kew (UK) are collected by experts following the protocols established by Kew (2005). Seeds of *Hordeum* and *Brassica* CWRs and achenes (hereafter referred to as seeds) of *Helianthus* CWRs were collected at maturity in their natural environment (Table 2.3). Seeds were equilibrated to 15 % RH and 15 °C. Subsequently they were stored under standard seed bank conditions at -20 °C (FAO/IPGRI, 1994) until use. Once out of the bank, the seeds were equilibrated to a dry room at 15 % RH and 15 °C for two days before use.

In this thesis, six seed lots (four species) of *Hordeum* CWRs, seven seed lots (three species) of *Brassica* CWRs and five seed lots (four species) of *Helianthus* CWRs (Table 2.3) were selected from different locations having different environments (precipitation and temperature). From the availability of the Millennium Seed Bank, the species were selected to cover a broad representation of their growth habitats (*Appendix* Figure A2.1).

2.2.2 Environmental data for CWRs site of origin.

The location of the seed lots was determined using GIS coordinates of the seed collection site. Historical annual mean temperature and mean monthly precipitation for the period 1950 –2000 (Hijmans *et al.*, 2005) were obtained from WorldClim with an accuracy of one kilometre from the GIS coordinates.

Data on the following environmental factors were extracted for further analysis (section 2.5): annual mean temperature (minimum, mean and maximum °C), mean monthly precipitation (mm) and the altitude (meters above the see level, m a.s.l.). The values were calculated as the mean precipitation and annual mean temperature, i.e. the sum of all 12-monthly means, divided by 12.

The month of germination was estimated for each seed lot by combining environmental factors (precipitation and temperature) and the calculated temperature thresholds to germination (section 2.5). It was assumed that germination occurred when the following two conditions were met after the month of seed dispersal: (1) the precipitation was above 20 mm (Freas & Kemp, 1983; Gutterman, 1993), and (2) the minimum environmental temperature exceeded base temperature (T_b explained in section 2.5) and the maximum environmental temperature was lower than ceiling temperature (T_c explained in *section 2.5*). The mean precipitation (mm) and mean temperature (°C) were extracted from the estimated month of germination for further analysis.

Table 2.3 Description of the maternal environment of the CWRs used in this study from the *Hordeum, Brassica* and *Helianthus* genus. The information was obtained from the seed collection site with GIS coordinates in WorldClim. The data of the accessions showed the mean monthly precipitation and historical annual mean temperature (T) between 1950 and 2000 as obtained from WorldClim.

			Annual mean temperature		
CWRs	**Location**	*Mean precipitation (mm)*	*Min T (°C)*	*Mean T (°C)*	*Max T (°C)*
Hordeum marinum	Kerkyra (Greece)	91.58	12.1	16.9	21.7
H. bulbosum	Calabria (Italy)	72.75	7.7	10.6	13.4
H. pusillum	Texas (USA)	69.83	13.4	19.6	25.8
H. bulbosum	Agios Nikolaos (Greece)	61.08	14.9	17.9	21.0
H. murinum	Peloponnisos (Greece)	59.50	11.5	15.8	20.2
H. murinum	Osh (Kyrgyz Republic)	33.67	4.8	11.0	17.2
Brassica rapa	Chur (Switzerland)	94.17	4.8	9.2	13.7
B. nigra	Dorset (England)	68.83	6.2	9.9	13.8
B. rapa	Memsault (France)	64.08	6.5	10.9	15.4
B. rapa subsp. *campestris*	Göle-Kars (Turkey)	40.84	-1.6	4.8	11.5
B. rapa subsp. *sylvestris*	Ait Marghad (Morocco)	26.80	3.8	12.1	20.4
B. tournefortii	Natrun-Alamin (Egypt)	4.83	14.3	20.7	27.1
B. rapa subsp. *sylvestris*	S. Oran (Algeria)	0.83	17.5	25.7	34.0
Helianthus glaucophyllus	North Carolina (USA)	120.75	3.6	9.5	15.5
H. angustifolius	North Carolina (USA)	99.50	9.5	16.2	22.9
H. angustifolius	Texas (USA)	85.17	12.5	19.0	25.6
H. argophyllus	Texas (USA)	72.17	17.5	21.9	26.4
H. pumilus	Colorado (USA)	37.42	-1.1	7.4	15.9

2.2.3 Crops

Hordeum vulgare: Seeds of *H. vulgare* (barley) genotypes were provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK, Germany).

The long-lived barley genotype, 2110, was grown under controlled conditions at 18/22 °C (night/day) (Table 2.4) in a glasshouse. Half of the plants were grown under non-limiting water availability (hereafter referred to as "control" treatment) in a glasshouse compartment and the other half were irrigated at 9 % of field capacity during the seed filling stage (hereafter referred to as "drought" treatment) in another compartment but in the same glasshouse. Soil moisture was determined by gravimetric measurements and used to calculate the amount of water to be added to the soil to maintain the desired level. The plants of both treatments, control and drought, were grown at the same time in the same glasshouse and the seeds were harvested in August 2013. In addition to these seed lots, one commercial seed lot of *H. vulgare* was purchased from B&T World Seeds (hereafter referred to as the "commercial seed lot").

Brassica oleracea: Seeds of two research genotypes (A12DHd and AGSL101) of *B. oleracea* were provided by the University of Warwick (UK). Both have the same genetic background but genotype AGSL101 has introgressions of two QTL (*RABA* and *SOG1*) from a second genotype (GDDH33) that confer higher vigour (Morris *et al.*, 2016). Furthermore, plants of both crop genotypes were grown under controlled conditions at 18/22 °C (night/day) (Table 2.4) in a glasshouse with two treatments: in one treatment the plants were grown under non-limiting water availability (hereafter referred to as "control" treatment). In the other treatment, the irrigation was controlled to maintain a leaf water potential of -1.0 MPa during the seed filling stage (hereafter referred to as the "drought" treatment). Three replicates of genotypes and watering treatments were grown at the same time in a randomised block design and harvested in August of 2014. In addition to these research genotypes, a commercial seed lot of *B. oleracea* was purchased from B&T World Seeds (hereafter referred to as the "commercial seed lot").

Helianthus annuus: Seeds of five sunflower genotypes were provided by Limagrain (France, hereafter referred to as Limagrain genotypes) (Table 2.4). They had a different genetic background and varied in oil composition and in the earliness of their production (Table 2.5). All Limagrain genotypes were grown to produce seeds in the field in Marchena (Sevilla, Spain) where the mean minimum temperature was around 16 °C and the mean maximum was 32 °C in 2014. Two treatments were applied to all Limagrain genotypes. One of the

treatment consisted of watering the plants with 3 to 4 irrigations before flowering and then 2 or 3 more irrigations during seed filling (hereafter referred to as “normal irrigation”). In the other treatment irrigation during seed filling was not applied (hereafter referred to as “stopped irrigation”) until 60 % of the lower leaves were dried. The watering was applied by drip irrigation to reduce the risk of fungal infection. In addition, a commercial seed lot of *H. annuus* was purchased from B&T World Seeds (hereafter referred to as the “commercial seed lot”).

Table 2.4 Description of the crop seed lots used in this study from the *Hordeum, Brassica* and *Helianthus* genus. The research crop genotypes of *Hordeum* and *Brassica* were grown in a glasshouse under controlled conditions (18/22 °C night/day), the Limagrain genotypes of *Helianthus* were grown in the field in Sevilla (Spain, 16/32 °C min/max). The commercial seed lots were grown in The Netherlands.

Crop species	Provider	Genotype	Growth conditions
Hordeum vulgare	IPK (Germany)	2110 control treatment	Glasshouse
H. vulgare	IPK (Germany)	2110 drought treatment	Glasshouse
H. vulgare	B&T World Seeds (France)	Commercial seed lot	Netherlands
Brassica oleracea	University of Warwick (UK)	A12DHd (low vigour) control	Glasshouse
B. oleracea	University of Warwick (UK)	A12DHd (low vigour) drought	Glasshouse
B. oleracea	University of Warwick (UK)	AGSL101(high vigour) control	Glasshouse
B. oleracea	University of Warwick (UK)	AGSL101(high vigour) drought	Glasshouse
B. oleracea	B&T World Seeds (France)	Commercial seed lot	Netherlands
Helianthus annuus	Limagrain (France)	A, normal irrigation	Field
H. annuus	Limagrain (France)	A, stopped irrigation	Field
H. annuus	Limagrain (France)	B, normal irrigation	Field
H. annuus	Limagrain (France)	B, stopped irrigation	Field
H. annuus	Limagrain (France)	C, normal irrigation	Field
H. annuus	Limagrain (France)	C, stopped irrigation	Field
H. annuus	Limagrain (France)	D, normal irrigation	Field
H. annuus	Limagrain (France)	D, stopped irrigation	Field
H. annuus	Limagrain (France)	E, normal irrigation	Field
H. annuus	Limagrain (France)	E, stopped irrigation	Field
H. annuus	B&T World Seeds (France)	Commercial seed lot	Netherlands

Table 2.5 Description of the seed oil composition differences between the genotypes of *Helianthus annuus* provided by Limagrain and the earliness of the flowering cycle.

Limagrain genotypes	Earliness	Oleic acid (%)	Linoleic acid (%)
A	Semi late	65	30
B	Semi early	30	65
C	Semi early	87	10
D	Semi late	35	60
E	Early	88	4

2.3 Seed morphology

Two techniques were used to study seed morphology. For the first, fifteen to 20 seeds of each seed lot in the *Hordeum* (Figure 2.1 A) and *Brassica* (Figure 2.1 B) genera were imbibed up to 24 h in water at room temperature (20 °C ± 2 °C). The seeds were then covered with a water-soluble compound (FSC22 Clear Frozen Section Media, Leica Biosystems, Nußloch, Germany) that encapsulates the seed for cryosection. Longitudinal cuts were performed in the seeds using a cryo-microtome (CM30505, Leica Biosystems, Nußloch, Germany) (Figure 2.1). It was difficult to consistently cut the same part of the seed, especially in rounded seeds such as *Brassica* seeds, therefore the measurements were taken in the widest part of the seed (Stuppy, W. personal communication, Figure 2.1 B). Images of the sectioned seeds were taken under a stereoscope (Stemi SV 11, Carl Zeiss, Oberkochen, Germany) with an attached camera (AxioCam HRc 412-312, Carl Zeiss, Oberkochen, Germany). The second technique was only applied to seeds of the *Helianthus* genus. Non-destructive morphological measurements were record by x-ray imaging analysis. One hundred seeds of each seed lot of the *Helianthus* genus were x-rayed (Figure 2.1 C) in a Faxitron digital x-ray mx 20 (Faxitron Bioptics, Arizona, USA). The measurements of the seed, in all species, were the thickness of the pericarp or seed coat and the embryo length. The embryo was measured longitudinally and the thickness of the seed coat was measured in the area where the seed was widest (i.e. midsection of the seed, Figure 2.1) (Stuppy, W. personal communication) in *Hordeum* and *Helianthus*. The endosperm length was also longitudinally

measured in endospermic seeds (*Hordeum* genus, Figure 2.1 A). Additionally, the distance from radicle to pericarp was measured in the *Helianthus* genus (Figure 2.1 C). The seeds were measured individually using Axiovision software (AxioVs40, Carl Zeiss Micro Imaging 2010, Germany), and the mean and coefficient of variation (CV) were calculated to use in further analysis. The thickness of the seed coat or pericarp and the distance from radicle to pericarp (in *Helianthus* seed lots) were divided by the mean embryo length to compare seed lots with different seed size.

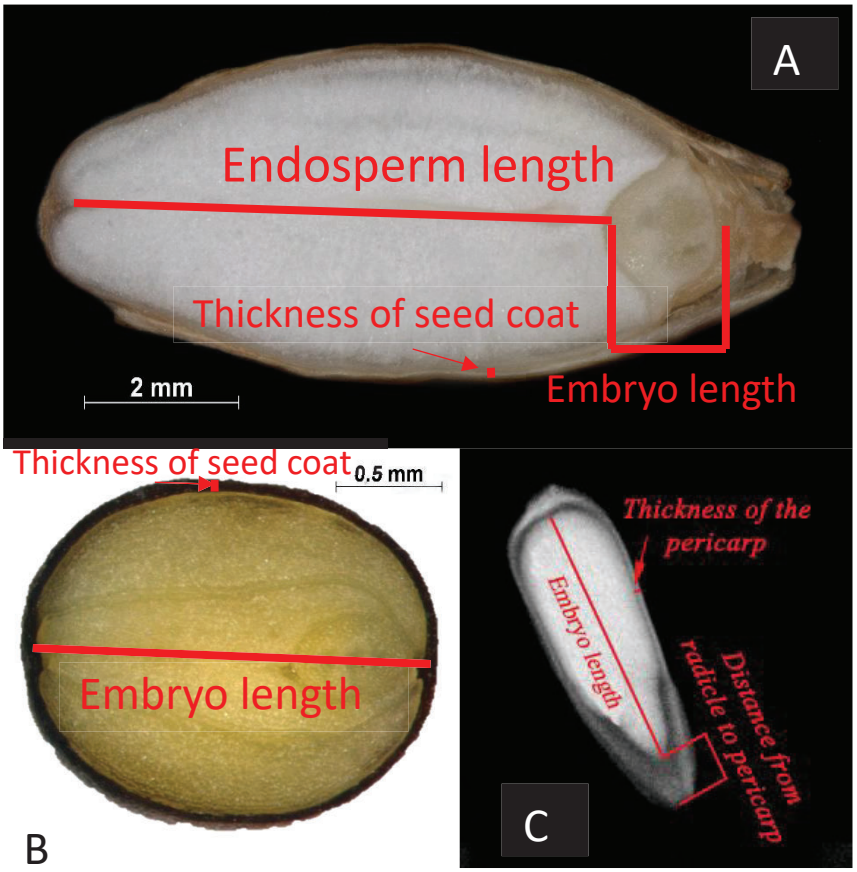


Figure 2.1 Example of seed sections of the crop seed lots, *Hordeum vulgare* (A) and *Brassica oleracea* (B) and x-ray image of *Helianthus annuus* (C).

2.3.1 Seed mass

Seed mass was quantified by weighing seeds individually on a precision balance (Mettler Toledo XP2U, Leicester, UK) capable of weighing a gram to 6 decimal places. In total 100 seeds were used for each species. The mean CV and normal distribution of the seed mass were calculated.

2.4 Seed germination

Seed germination was recorded in all seed lots listed in Table 2.3 and Table 2.4. Three replicates of 25 seeds in the CWR seed lots and four replicates of 25 seeds for the crops were used. The seeds were sown onto two layers of moist germination test paper (test seed paper; 160g/m^2, 90 mm diameter, Fisher Scientific UK) in Petri dishes (90 mm diameter, Fisher Scientific, UK). Germination experiments were conducted under a range of constant temperatures from 5 to 45 °C ± 2 °C (Table 2.6). Extra germination experiments were needed at 42 °C, when the final germination dropped from nearly 100 % to 0 % between 40 and 45 °C. Additionally, germination experiments at 0 °C were also performed when the germination rate ($1/t_{50}$, see Section 2.5) and final germination between 15 to 5 °C was the same. The Petri dishes were placed in a sealed clear plastic bag and incubated at the relevant temperature with a 12-hour photoperiod (radiometric flux density of 50-100 W/m^2, Series 1A Cool incubators, LMS, Kent UK).

In addition, germination was recorded at different water potentials using distilled water (0 MPa) and four solutions of PEG 8000 (previously described in section 2.1.1) of -0.3, -0.5, -0.8 and -1.0 MPa. The seeds were incubated at a single constant temperature between 15 and 30 °C (Table 2.6) appropriate for the species concerned (close to the optimal temperature, when seed germination was fastest, see section 2.6). The exception was *B. nigra* due to the limited number of seeds available. In this species germination was recorded under constant temperatures on distilled water and not at different water potentials.

The volume of PEG solutions for each Petri dish (7 mL) was calculated following the Equation 2.1 of Hardegree and Emmerich (1990), who suggest that PEG molecules could be excluded from the fibres of the germination paper to effectively concentrate the solution. Thus, a ratio of 7:2 volume of the solution to weight of the dry germination paper was used to minimise the effect on PEG concentration. The same volume of 7 mL was used for both PEG and water in all experiments.

A seed was considered to be germinated when the radicle emerged through the seed surrounding layers by 2 mm in length. The frequency of germination recording was based on the rate of germination of each species. For the faster germinating *Brassica* CWRs and all the crops, germination was recorded every

two hours during the first 36 hours and then every three to four hours. For the slower germinating seeds of *Hordeum* and *Helianthus* CWRs, seed germination was recorded twice per day (morning and afternoon) during the first three days and then once per day thereafter. A germination experiment was considered finished when the germination did not increase over a period of 5 days or 2 weeks for the faster and slower germinating seeds, respectively. Non-germinated seeds were subjected to a cut test and firm, mouldy, empty or infested seeds were identified. The mean germination percentages were calculated on the basis of the total number of full seeds sown, i.e., empty and infested seeds were excluded based on the cut test.

At the end of experiments with -0.3, -0.5, -0.8 and -1.0 MPa, non-germinated seeds that were still firm were washed with distilled water and transferred to a new Petri dish with germination test paper and 7 mL of distilled water at the same temperature. If the seeds did not germinate, the seed coat was removed following the protocol determined by ISTA (2003) and the viability was assessed with 1 % triphenyl tetrazolium chloride (TTC, section 2.1.4) in the darkness at 30 °C (ISTA, 2003) for 18 hours. The seed embryo was considered viable when it was fully stained with red or pink colour. The mean germination percentages were adjusted according to the viable seed embryos determined on the TTC test, i.e., non-fully stained embryos were excluded.

Table 2.6 Description of the seed germination experiments (temperature and water potential) on CWRs and crops of the three genera studied: *Hordeum*, *Brassica* and *Helianthus*. *Seed lots with seed dormancy

CWRs	Location	Temperatures used for germination (°C)	Water potential used for germination (MPa)
Hordeum marinum	Greece	0, 5, 10, 15	0, -0.5, -0.8, -1.0 at 15 °C
H. bulbosum	Italy	5, 10, 15	
H. pusillum	Texas (USA)	5, 10, 15, 20	
H. bulbosum	Greece	5, 10, 15	
H. murinum	Greece	5, 10, 15, 20	
H. murinum	Kyrgyz Republic	5, 10, 15	
Brassica rapa	Switzerland	5, 15, 20, 25	0, -0.5, -0.8, -1.0 at 20 °C
B. nigra	England	10, 15, 20, 25	No data
B. rapa	France	15, 20, 25	0, -0.5, -0.8, -1.0 at 20 °C
B. rapa subsp. *campestris*	Turkey	5, 10, 15, 20, 25, 30, 35, 40	0, -0.3, -0.5 at 30 °C
B. rapa subsp. *sylvestris*	Morocco	15, 20, 25, 30, 35, 40	0, -0.3, -0.5, -0.8, -1.0 at 25 °C
B. tournefortii	Egypt	10, 15, 20, 25, 30, 35, 40	0, -0.3, -0.5, -0.8 at 20 °C
B. rapa subsp. *sylvestris*	Algeria	15, 20, 25, 30, 35	0, -0.5, -0.8, -1.0 at 20 °C
**Helianthus glaucophyllus*	North Carolina (USA)	10, 20, 25, 30, 35	0, -0.3, -0.5 at 20 °C
**H. angustifolius*	North Carolina (USA)	10, 15, 20, 25, 30, 35	
**H. angustifolius*	Texas (USA)	10, 20, 25, 30, 35	
H. argophyllus	Texas (USA)	5, 10, 15, 20, 25, 30, 35	0, -0.3, -0.5, -0.8, -1.0 at 20 °C
**H. pumilus*	Colorado (USA)	5, 10, 15, 20, 25, 30, 35	0, -0.3, -0.5 at 20 °C

Table 2.6 Continuation

Crop species	Genotype	Temperatures used for germination (°C)	Water potential used for germination (MPa)
Hordeum vulgare	2110 control	5, 10, 20, 25, 30, 35	0, -0.5, -0.8, -1.0 at 20 °C
H. vulgare	2110 drought		
H. vulgare	Commercial seed lot	5, 10, 15, 20, 25, 30	0, -0.3, -0.5, -0.8, -1.0 at 20 °C
Brassica oleracea	A12DHD (low vigour) control	10, 20, 25, 30, 35, 40	0, -0.3, -0.5, -0.8 at 25 °C
B. oleracea	A12DHD (low vigour) drought		
B. oleracea	AGSL101 (high vigour) control		
B. oleracea	AGSL101 (high vigour) drought		
B. oleracea	Commercial seed lot	10, 15, 20, 25, 30, 35, 40	
Helianthus annuus	A normal irrigation	10, 20, 25, 30, 35	0, -0.3, -0.5, -0.8 at 20 °C
H. annuus	A stopped irrigation		
H. annuus	B normal irrigation	10, 20, 25, 30, 35, 40	
H. annuus	B stopped irrigation		
H. annuus	C normal irrigation	10, 20, 25, 30, 35	
H. annuus	C stopped irrigation		
H. annuus	D normal irrigation		
H. annuus	D stopped irrigation		
H. annuus	E normal irrigation		
H. annuus	E stopped irrigation		
H. annuus	Commercial seed lot	10, 15, 20, 25, 30, 35, 40, 42	

2.4.1 Normal seedlings

Normal seedlings were recorded twice per week from the germinated seeds. A seedling was considered normal when all the essential structures (roots, hypocotyls and cotyledons) were completely developed, healthy and in proportion to each other (ISTA, 2017). Moreover, mouldy cotyledons or roots, non-green cotyledons or hypocotyls were considered abnormal seedlings. Percentage normal seedlings (S %) were calculated on the basis of the total number of full seeds sown, i.e., empty and infested seeds were excluded based on the cut test.

2.4.2 Germination of dormant seeds

The dormant species (CWRs of *Helianthus* except *H. argophyllus*) were germinated in several concentrations of GA_3 (0.5, 1.0, 2.5, 5, 7.5 and 10.0 mM) at pH 7, (section 2.1.2) to select the concentration at which the maximum germination percentage was obtained. Two controls were used, one in the phosphate citrate buffer used to prepare GA_3 (section 2.1.2) and the other in water. In addition, for each GA_3 concentration scarified and non-scarified seeds were examined. Scarification was performed at the root end of the pericarp with a scalpel by removing or breaking a section of the pericarp to help the emergence of the radicle. Seed germination experiments were prepared as described in section 2.5. However, in this case, PEG solutions were made using 5 mM GA_3 solutions (selected as the optimal concentration from the preliminary results, Chapter 3) to perform the germination experiments at -0.3 and -0.5 MPa.

2.5 Thermal time, hydro time and hydrothermal time models

Germination progress over time is represented as a sigmoidal curve (fitted using the Boltzmann distribution, Equation 2.2) that differs with germination conditions. Seed germination (radicle emergence) is quantified as germination rate (GR) and final germination percentage (%). GR is the reciprocal of time ($1/t_{50}$) taken to reach 50 % of germination based of the viable population of seeds. The parameters studied were: cardinal temperatures, thermal time, base water potential, hydro time and hydrothermal time.

$$y = A_2 + \frac{A_1 + A_2}{1 + \exp\left(\frac{x - x_0}{dx}\right)} \qquad \text{Equation 2.2}$$

Where y is the germination progress and x is the time. A_1 is the germination on the day cero, A_2 is the final germination, x_0 is the time when the germination is 50 % of the final germination, dx is the slope of the sigmoidal curve.

2.5.1 Cardinal temperatures

Species exhibit minimum, optimum and maximum temperatures for germination (Bradford, 1995). Two methods are described to analyse the cardinal temperatures for seed germination based on rates. The first was obtained by García-Huidobro *et al.* (1982) who calculated the cardinal temperatures (Figure 2.2A): base temperature, T_b (the lowest temperature at

which germination can proceed to completion); optimal temperature, T_o (highest GR); and ceiling temperature, T_c (highest temperature at which germination can proceed to completion). García-Huidobro *et al.* (1982) used regression lines when GR was plotted against temperature. The range of temperatures between the T_b and T_o is the sub-optimal range, and between T_o and T_c is the supra-optimal range.

The second method is the repeated probit analysis that combines all the germination data over time at a range of temperatures into a common regression and is assumed to have a common T_b (Covell *et al.*, 1986) and different T_c for each percentile of the seed population (Figure 2.2A). The slope and the intercept are calculated by the ordinary least squares method (Dahal et al., 1990) and then different T_b values are used in Equation 2.3 until the residual mean square of the regression is minimized (Dahal *et al.*, 1990).

2.5.2 Thermal time model

The germination response to accumulated temperature can be modelled using the thermal time approach (Covell *et al.*, 1986; Ellis *et al.*, 1986; Ellis *et al.*, 1987; Pritchard & Manger, 1990; Bradford, 1995; Hardegree, 2006). Thermal time (θ_T) is the accumulated heat units required for a given percentile (*g*) to complete the germination process in chronological time (e.g. °C hours) (Bradford, 1995). In the sub-optimal range of temperatures, $\theta_{T(g)}$ is the thermal time for each germination percentage, g. T is the temperature and T_b is the base temperature defined above, finally t_g is the time to complete seed germination in a given percentage g (Equation 2.3)

$$\theta_{T(g)} = (T - T_b) \cdot t_g \qquad \text{Equation 2.3}$$

In non-dormant seeds T_b is assumed equal for all seeds (García-Huidobro *et al.*, 1982; Ellis *et al.*, 1987; Bradford, 1995). Thus, "$(T\text{-}T_b)\cdot t_g$" is represented as K (constant intercept of Equation 2.5).

In the supra-optimal range of temperatures, θ_{Tsupra} is the thermal time, T is the temperature and T_c is the ceiling temperature defined above, finally t_g is the time to complete seed germination in a given percentage g (Equation 2.4)

$$T_{c(g)} = T + (\theta_{Tsupra}/t_g) \qquad \text{Equation 2.4}$$

In the supra-optimal range of temperatures, θ_{Tsupra} is assumed equal for all seeds (Covell *et al.,* 1986). Thus, "(T + (θ_{Tsupra}/t_g)" is represented as K (constant intercept of Equation 2.6).

Probit analyses are applied to estimate the parameters for sub and supra-optimal range of temperatures using GR (Bradford, 1995). Three methods have been used:

1) Reciprocal of the slope from the regression line (Figure 2.2A)

2) Probit analysis from cardinal temperatures (Bradford, 1995)

Sub-optimal thermal time (Covell *et al.*, 1986; Ellis *et al.*, 1986; Bradford, 1995)

$$\text{Probit}(g) = K + (\log \theta_{T(g)})/ \sigma_{\theta T} \qquad \text{Equation 2.5}$$

Supra-optimal thermal time (Covell *et al.*, 1986; Pritchard *et al.*, 1996)

$$\text{Probit}(g) = K + T_c/ \sigma_{\theta T} \qquad \text{Equation 2.6}$$

3) Repeated probit analysis to obtain the best fit. θ_T and T_c are estimated by varying T_b and θ_{Tsupra} until the least residual mean square (best fit) is obtained.

In Equations 2.5 and 2.6, K is the intercept constant when θ_T is zero. θ_T may be normal or log-normally distributed (Hardegree, 2006) and σ is the inverse of the probit regression line of the response to θ_T or the standard deviation of log θ_T, i.e. distribution of the θ_T in the population of seeds (Covell *et al.*, 1986; Bradford, 1995).

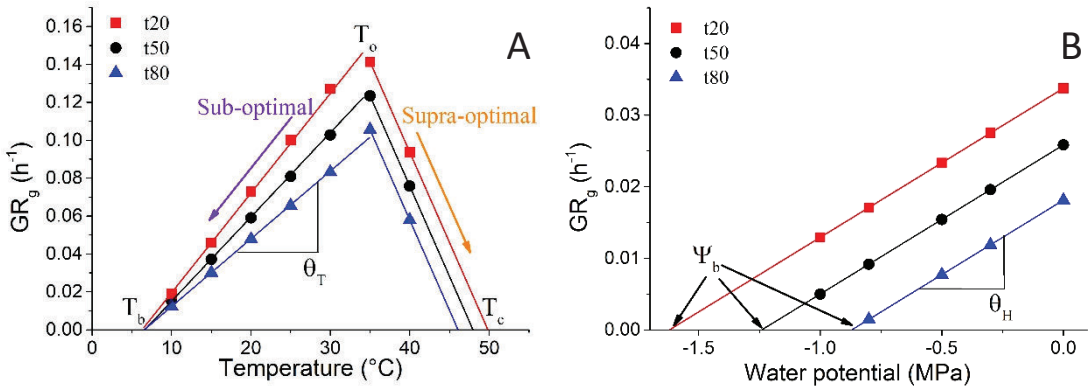


Figure 2.2 Thermal and hydro time models. Example of how the germination rate (GR) can be plotted as a function of **A)** temperature; the base temperature (T_b), optimal temperature (T_o) and ceiling temperature (T_c) are calculated from two regression lines (sub and supra optimal range of temperatures) for three percentiles (t20 in red, t50 in black and t80 in blue) The inverse of the slope is the thermal time (θ_T). **B)** water potential; GR can be plotted against water potential and the base water potential (Ψ_b) is shown by the intersection of the regression lines with the X axis for three percentiles (t20 in red, t50 in black and t80 in blue). The inverse of the slope is the hydro time (θ_H).

2.5.3 Base water potential

Water potential is the other factor, besides temperature, which has been studied, and was simulated using solutions of polyethylene glycol (PEG) 8000 (Fisher Scientific, UK). Two parameters can be quantified, base water potential (Ψ_b, which is the lowest water potential at which germination can proceed to completion), and the hydro time (explained below). A linear relationship between GR and water potentials is used to calculate Ψ_b (i.e. Ψ_b is the intercept when GR = 0; Figure 2.2B). The repeated probit analysis is another method to determine Ψ_b and the hydro time (explained below) where Ψ_b is considered to be different for each percentile of the population, but with a common slope (i.e. same hydro time) (Figure 2.2B) (Gummerson, 1986; Bradford, 1990). Several values are assigned to the θ_H until the best fit is obtained, and finally Ψ_b is recalculated (Bradford, 1995).

2.5.4 Hydro time model

Hydro time (θ_H) has been used to describe the influence of water potentials on germination and is measured in units of MPa hours or days (Equation 2.7) (Gummerson, 1986; Bradford, 1995).

$$\Psi_{b(g)} = \Psi - (\theta_H/t_g) \quad \text{Equation 2.7}$$

where Ψ is the water potential and $\Psi_{b(g)}$ is the base water potential for each germination percentage and t_g is the time to complete seed germination in a given percentage g time. Finally, θ_H is constant for the entire population (Gummerson, 1986; Bradford, 1995), thus "$\Psi - (\theta_H/t_g)$" is represented as K (constant intercept of Equation 2.8)

Probit analyses are applied to estimate the parameters for sub and supra-optimal range of temperatures using GR (Bradford, 1995). There are three different methods to calculate θ_H (Bradford, 1995):

1) Reciprocal of the slope from the regression line (Figure 2.2B).

2) Probit analysis (Bradford, 1995) describes the effect of the water potential:

$$\text{Probit}(g) = K - (\Psi_{b(g)})/\sigma_{\theta H}) \quad \text{Equation 2.8}$$

3) Repeated probit analysis to obtain the best fit (as mentioned before in the section 2.5.2), and then Ψ_b is recalculated.

In Equation 2.8, K is the intercept constant when θ_H is zero and σ is the standard deviation of θ_H or the inverse of the slope of the response to θ_H (Bradford, 1995).

2.5.5 Hydrothermal time model

The hydrothermal time (θ_{HT}) model provides understanding of how physiological and the environmental factors temperature and water potential interact to regulate the germination behavior of seed populations (Bradford, 1995; Bradford, 2002). θ_{HT} describes the progress toward seed germination under various combinations of water potential and temperature (Gummerson, 1986; Allen *et al.*, 2000). This model assumes that the accumulation of heat units occurs in the sub-optimal range of temperatures (between T_b and T_o) (Gummerson, 1986; Bradford, 1995). The differences in time to germination could be due to variation in T_b and Ψ_b, or θ_{HT} required for germination (e.g. MPa °C h):

$$\theta_{HT} = ((T-T_b)\cdot(\Psi - \Psi_{b\,(g)}))\cdot t_g \quad \text{Equation 2.9}$$

An increase in T_b, Ψ_b or θ_{HT} will reduce the germination rate (Allen *et al.*, 2000). In Equation 2.6 (Gummerson, 1986; Bradford, 1995) it is assumed that

temperature and water potential are independent, which means that the possible interactions are not considered (Larsen *et al.*, 2004). However other studies have proved that these interactions exist (Kebreab & Murdoch 1999b) and this will be discussed in Chapter 8.

2.6 Quantification of seed oil content

Whole seeds were used to measure the oil content and moisture content using time domain nuclear magnetic resonance spectroscopy (TD-NMR, the minispec NMR analyser mq20, Bruker, Coventry, UK) (Borisjuk *et al.*, 2011). For each population, the oil content was quantified in three replicates of seeds previously stored at 15 % RH, however, especially in small seeds (CWRs) only one replicate was possible. For seeds of the *Helianthus* crop, an 18 mm probe assembly (H20-18-25A1) was used to accommodate the larger sized seeds and each replicate contained 2.5 to 3.0 g of seed. For all other species, a 10 mm probe assembly (H20-10-25AVGX4) was used and each replicate containing 0.55 to 0.60 g of seed. Samples were analysed in a Bruker mq20 minispec, with a 0.47 Tesla magnet (20 MHz proton resonance frequency) operating at 40 °C. The method consisted of the acquisition of 16 scans with a recycle delay of 2 seconds (Figure 2.3). For each sample, a calibration method with seeds of *Brassica oleracea* of known oil and water content was used to quantify the oil content, and a sunflower oil calibration was used to confirm the oil content data. Data are expressed as % oil content (w/w).

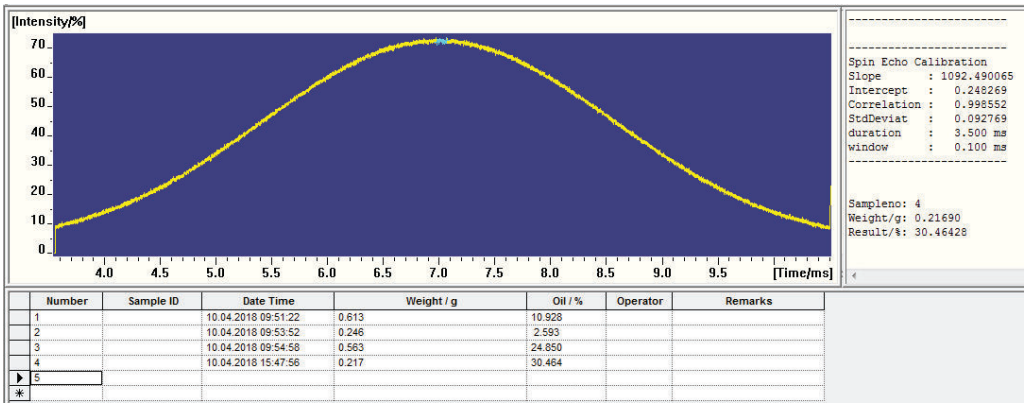


Figure 2.3 Example of a run sample in the NMR to measure oil content.

2.7 Seed longevity

Seed longevity was calculated on the two irrigation treatments of Limagrain genotype B of *Helianthus annuus* (see section 2.2.3) because it was the *Helianthus* genotype in which maternal environment has more impact on seed functional traits.

2.7.1 Adjusting and measuring seed moisture

Different RHs (30 %, 45 %, 60 % and 75 %) were obtained using various solutions of lithium chloride (LiCl, section 2.1.3) to adjust the seed moisture content and for artificially ageing. Between 20 to 50 g of LiCl (≥ 99 %, 10491241, Fisher Scientific, UK) were added slowly to distilled water. One litre of solution was poured into sealed boxes (electrical enclosure boxes, 300 x 300 x 130 mm, Ensto Ltd, Southampton, UK) and placed at 20 °C, 30 °C and 40 °C ± 2 °C. The RH did not change during the ageing time and thus did not require adjustment (see section 2.1.3).

The target moisture contents (MCs) of the seeds during ageing were predicted from information on equilibrium, seed oil content (analysed in Chapter 3) and temperature given in the Seed Information Database website (SID). The MC estimates were fed into the viability equation constants for *H. annuus* (Ellis & Roberts, 1980) as reported in SID. Prior to ageing, the seeds were equilibrated over salt solutions of LiCl at 20 °C until they reached the predicted seed MC (Table 2.7). The equilibrium relative humidity (eRH) was monitored weekly on three samples of seeds (approximately 20-30 seeds each) using a hygrometer (sensor housed in an AW-D10 water activity probe, HygroPalm, Rotronic instruments Ltd, Crawley, UK). The seeds were equilibrated within four weeks. The boxes containing the equilibrated seeds were moved to the temperature chambers at temperatures of 20 ℃, 30 ℃ and 40 ℃. For example, seeds equilibrated at eRH of 63.9 % at 20 °C were transferred to the temperature chamber at 40 ± 2 °C in a LiCl solution of 75 % RH (Table 2.7). Both normal and stopped irrigation seed lots were placed in the same sealed box and thus aged under exactly the same conditions. Overall, seeds were aged at three constant temperatures in combination with up to four RHs.

Table 2.7: Artificial ageing conditions for both seed lots of *Helianthus annuus* (normal and stopped irrigation). T is the constant temperature (°C) during seed ageing, RH is the constant relative humidity (%), MC is the estimated moisture content in wet weight basis (%) predicted for the *H. annuus* seeds for each condition and eRH is the equilibrium relative humidity at which the seeds were pre-equilibrated at 20 °C for four weeks. The measurements of the hygrometer had a standard error of ± 2 % RH.

Ageing T (°C)	Target RH (%)	Predicted MC (%)	eRH (%) at 20 °C
40	75.0	9.0	63.9
	60.0	7.4	48.6
	45.0	6.1	35.6
	30.0	4.8	23.3
30	75.0	9.6	69.1
	60.0	7.0	54.5
	45.0	6.5	39.6
20	75.0	10.3	75.0
	60.0	8.6	60.0

Seed MC was measured at the beginning and at the end of the ageing experiments to observe possible variations. Thus, five to 10 seeds were weighed individually in a precision balance (XP2U, Mettler Toledo, UK) and dried at 103 ± 2 °C for 17 hours (ISTA, 2017). The dry seeds were placed in a container with silica gel to cool and keep dry prior to weighing. The percentage MC on a wet weight basis was calculated as follows:

$$MC\ (\%) = \frac{wet\ seed\ weight - dry\ seed\ weight}{wet\ seed\ weight} * 100 \qquad \text{Equation 2.10}$$

The number of seeds was limited therefore it was not possible to measure MC during the ageing process (i.e. at all time intervals used).

2.7.2 Seed ageing

Seed longevity can then be predicted from controlled storage conditions with the Equation 2.11 (Ellis & Roberts, 1980):

$$v = Ki - {}^{p}/_{10^{K_E - C_W log MC - C_H T - C_Q T^2}} \qquad \text{Equation 2.11}$$

Where v is the viability, in probit units, after p days of seed storage and Ki is the initial viability (germination) of the seed lot in probit units. K_E and C_W are moisture constants and C_H and C_Q are temperature constants. The moisture content, MC, is measured for each seed lot and each ageing conditions and

expressed on a wet weight (above described). The temperature, T, is expressed in degrees Celsius (Roberts & Ellis, 1989).

Seeds were aged for five time intervals (t_1, t_2, t_3, t_4 and t_5) at each ageing condition (Table 2.8). The days of ageing were initially estimated using Equation 2.11 of Ellis and Roberts (1980) assuming the viability constants for sunflower publicly available in SID. The time intervals for ageing were chosen to give the following viabilities: t_1 (100 %); t_2 (85 %); t_3 (60 %); t_4 (50 %); and t_5 (25 %) (Table 2.8). To confirm the values, 10 to 15 seeds were germinated at 20 ºC after the target times to observe whether the actual viability was similar to the expected viability in each interval (Table 2.8). For example, seed viability was predicted to fall to 50 % after 14 days at 40 ºC and 75% RH (i.e., t_3). However, the viability equations tended to underestimate the longevity of the seeds and longer periods of time were needed between time intervals at all ageing conditions in both seed lots (*Appendix* Table A7.1).

Table 2.8: Description of the time intervals (t_1, t_2, t_3, t_4 and t_5) analysed during the ageing experiment at three constant temperatures and relative humidities (RH). The days of ageing were estimated using Equation 2.11 assuming the viability constants reported by Ellis *et al.* (1988). The seeds of the time intervals t_1, t_2 and t_4 (shaded cells) were germinated at 10, 20 and 25 °C and at 0, -0.3 and -0.5 MPa at 20 °C. The seeds of the intervals t_3 and t_5 (non-shaded cells) were germinated at 20 °C only.

Time intervals (expected viability, %)	**40 °C**				**30 °C**			**20 °C**	
	75 % RH	60 % RH	45 % RH	30 % RH	75 % RH	60 % RH	45 % RH	75 % RH	60 % RH
	Days of ageing after equilibrium								
t_1 (100 %)	0	0	0	0	0	0	0	0	0
t_2 (≈85 %)	7	14	98	165	21	105	130	54	136
t_3 (≈60 %)	11	25	115	182	40	122	156	94	225
t_4 (≈50 %)	14	38	129	205	52	159	206	227	> 350
t_5 (≈25 %)	20	49	151	270	70	200	> 350	281	> 400

2.7.3 Seed germination testing

For each ageing condition, a sample of 75 seeds was taken at each time interval to record germination at 20 °C (Table 2.8) to calculate the viability based on percentage seed germination (radicle emergence). Another sample of 375 seeds was taken at t_1, t_2 and t_4 to perform a thermal time (θ_T) analysis from

seeds germinating at 10 °C, 20 °C and 25 °C ±2 °C and hydro time (θ_H) analysis from seeds germinating at 0, -0.3 MPa and -0.5 MPa at 20 °C. These latter analyses were used to see if the seed population θ_T and θ_H and their thresholds changed during ageing. They represent approaches to assessing germination performance under thermal and drought stress (i.e. water potentials).

Prior to the seed germination experiments, all the seed samples were transferred to 15 % RH and 15 °C for two days. In this way, direct comparisons could be made between ageing conditions. For each germination experiment, three replicates of 25 seeds were placed to germinate following the methodology explained in Chapter 2 section 2.5. Additionally, the initial viability (before seeds were pre-equilibrated at 20 °C) of the seed lots was included as a control (t_f), to test whether pre-equilibration at 20 °C affected the viability of the seeds. The non-germinated seeds for each experiment were mouldy (i.e. non-firm) and it was not possible to perform a tetrazolium test (see section 2.1.4) to assessed whether the seed was alive or death.

The proportion of normal seedlings (PNS %) was calculated using the number of normal seedlings (PNS) and the total number of germinated seeds (G) for each time interval and ageing condition.

$$PNS\ \% = \frac{Number\ of\ normal\ seedlings * 100}{Number\ of\ germinated\ seeds} \qquad \text{Equation 2.12}$$

2.8 Data analysis

The normal distribution of the seed mass (calculated with D'Agostino K^2 test) and the seed germination sigmoidal curves (modelled using a Boltzmann fit) were calculated using Origin 9.1 software (Origin Corporation, 2013). ANOVA analysis followed by Tukey analysis were performed to observe significant differences among seed lots. To compare two means (i.e. final water content or the rate of imbibition) the student's t-test (t-test) was used. The percentages were arcsine transformed prior to data analysis. Additionally, the seed morphology measurements (embryo length, thickness of the seed coat, etc.) and mass were compared between populations using the Fisher test (F-test) and student's t-test (t-test). Correlations between seed traits and the maternal environment of CWRs were plotted in a scatter matrix and then linear regressions were performed.

GenStat 12.1 software (VSN International Ltd, 2009) was used to perform probit analysis on the data obtained from thermal, hydro and hydrothermal time models.

3 CHAPTER 3: SEED CHARACTERISATION OF *HORDEUM*, *BRASSICA* AND *HELIANTHUS* GENERA

3.1 Introduction

Seeds of three crops, *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus* are characterised in this chapter in addition to their CWRs. These crops are included in the crop list of Annex 1 of the International Treaty on Plant Genetic Resources for Food and Agriculture (FAO, 2009). Angiosperms constitute approximately 90 % of the plants in the world. The last update on angiosperm phylogeny shows two main groups based on the numbers of cotyledons: Monocotyledoneae (one cotyledon) and Dicotyledoneae (more than one cotyledon).

Due to genetic variability and environmental adaptation, the morphology of the seeds will differ between wild populations as a result of natural selection in each environment (Salazar & Quesada, 1987; Ginwal *et al.*, 2004). Seed mass is one trait widely studied in relation to seed germination (discussed in the following chapters) and the maternal environment (Fenner, 1992; Murray *et al.*, 2004; Harel *et al.*, 2011). For example, the seed mass of *Hordeum spontaneum* was related to the maternal environment and the plant growth rate: plants growing in dry environments with a higher relative plant growth rate produced smaller seeds than plants growing in wetter environments (Elberse *et al.*, 2003). However, seeds also have other traits such as embryo length, endosperm length, thickness of the seed coat and oil content that are also important when characterising a seed population. These morphological characters have been used to resolve taxonomic problems (Whiffin & Tomb, 1972; Zeng *et al.*, 2004; Kasem *et al.*, 2011; Gandhi *et al.*, 2013). Seed morphology can also influence the permeability of the seeds to water. For instance, the seed coat can prevent water uptake due to impermeable layers. This behaviour has been linked to seed coat colour in several legumes species (Maass, 2006), *Arabidopsis* mutants (Debeaujon *et al.*, 2000) and *Brassica napus* seeds (Zhang *et al.*, 2008).

Embryo size is influenced by the maternal environment. López-Castañeda *et al.* (1996) discussed the importance of embryo length and embryo mass of cereals (barley, triticale, wheat and oat) in determining seedling emergence. The

main finding was that seeds with large embryos emerged earlier than seeds with shorter embryos. Weiss *et al.* (2013) measured several seed characteristics in *Helianthus annuus* including the distance from the radicle tip to the outside of the pericarp and found that the wild *Helianthus annuus* had a longer distance, when the distance was divided by the seed length, than seeds from the crop seed lot. They suggested that crop seeds will germinate faster due to the shorter distance the radicle needs to elongate before radicle emergence. Therefore, it was hypothesised that *Helianthus* species with shorter distances between the radicle tip and the outside of the pericarp germinate faster than species with longer distances. The maternal environment also had an impact on the embryo length of Apiaceae where plants growing in dry regions produced seeds with larger embryos than plants growing in wetter regions (Vandelook *et al.*, 2012).

Seed oil is another characteristic explored in seeds of the *Brassica* and *Helianthus* genera. Previous studies have shown the influence of the maternal environment on oil content. For example, high light intensities during plant growth positively influenced seed size and oil content of *Arabidopsis* seeds (Li *et al.*, 2006). González Belo *et al.* (2014) analysed seeds of *H. annuus* crop genotypes and found seeds with high amounts of linoleic acid were able to germinate at lower temperatures than those with lower amounts. Furthermore, the potential for improving the quantity of oil in sunflower has been explored using two wild *Helianthus* species (*H. anomalus* and *H. deserticola*) with seeds that have higher oil content than the crop *H. annuus* (Seiler, 2007). The oil content is also affected by seed mass. Severino *et al.* (2015) found the oil content was higher in larger seeds than smaller seeds of *Ricinus communis* due to the reduction of the relative weight of the seed coat. However, other study found increases in seed mass were related to thicker seed coats (Lacey *et al.*, 1997). These hypotheses are tested for the three genera studied in this Chapter.

Several studies have identified fungal infection of oilseeds resulting from inadequate seed storage conditions, specifically high humidity (Robertson *et al.*, 1984; Roberts, 1986; Bhattacharya & Raha, 2002; Kakde *et al.*, 2012). These fungi can adversely affect seed germination and/or oil composition (Robertson *et al.*, 1984; Bhattacharya & Raha, 2002). Many field and storage fungi have been found in sunflower seeds after a period of storage at high moisture content (Roberts, 1986), especially *Aspergillus* and *Rhizopus* species (Bhattacharya &

Raha, 2002). The seeds of Limagrain genotypes analysed in the current study were infected by a fungus in the embryo. Seeds were screened to determine the extent of this infection and the impact of the fungus on seed germination is analysed in section 3.3.3.

Details of seed germination are not reported in this chapter as they are reported separately for each genus in subsequent chapters. Nonetheless, seed dormancy is an important seed trait that involves aspects of morphology such as seed coat, embryo or pericarp (Bewley, 1997; Baskin & Baskin, 1998) and these are included here. Seed dormancy is defined as failure of a viable seed to germinate even under non-limiting conditions i.e. air and water availability and optimal temperature (Baskin & Baskin, 1998). Dormancy may remain until suitable conditions for seedling survival have been reached (Bewley *et al.*, 2013). The combination of mechanical and biochemical actions in the seed coat or pericarp can affect the dormancy of the seed (Maeda & Ungaro, 1985). The mechanical effect of the pericarp may prevent radicle protrusion and interferes with water imbibition (impermeability) (Seiler, 1992). The permeability of the pericarp to water was therefore studied in dormant seeds of *Helianthus* CWRs in this chapter.

Previously, investigations on CWRs have focussed on their genetic variability and their tolerance to abiotic and biotic stress to investigate their potential use in plant breeding (Hajjar & Hodgkin, 2007). However, there are few comparative studies of seed traits such as thickness of the seed coat, embryo length or endosperm length of crops and their wild relatives. The objectives of this chapter are:

- To describe and compare the variability of seed traits in crops and their wild relatives of three genera *Hordeum, Brassica* and *Helianthus* (Chapter 2, Table 2.3 and 2.4).
- To determine the influence of the environment of seed collection site on these seed traits of CWRs
- To discuss the impact of limiting irrigation to the mother plant during seed filling of crop genotypes.
- To investigate whether *Helianthus* CWR seeds have impermeable seed coats and the extent of fungal infection in the study samples.

3.2 Materials and Methods

3.2.1 Seed material

A total of 18 wild seed lots (described in Chapter 2, Table 2.3) were used in this study to represent CWRs of *Hordeum, Brassica* and *Helianthus.* In addition, 19 crop seed lots of these genera (described in Chapter 2, Table 2.4) were also studied for comparison with the CWRs. A complete description of the seed lots is given in Chapter 2 section 2.2.1 and 2.2.3.

3.2.2 Total seed germination

In the subsequent chapters, seed germination for each genus will be discussed in relation to thermal and hydro time models. In this chapter total seed germination was used to characterise the seed lot. Total seed germination was calculated as the highest germination percentage at specified temperatures (*Appendix* Table A3.1). The seeds were imbibed at several temperatures (see Chapter 2, Table 2.6) on germination paper soaked with water. For the CWRs, three replicates, and the crops, four replicates, of 25 seeds each were used. The mean germination percentage was calculated on the basis of the total number of full and firm seeds sown (i.e., excluding empty and infested seeds based on a cut test).

3.2.3 Fungal infection

To identify and calculate the level of fungal infection in the seeds of *H. annuus* Limagrain genotypes, 100 seeds of each crop genotype and treatment were x-rayed and the presence of fungus was identified.

3.2.4 Seed morphology

Two techniques were used to study seed morphology (see Chapter 2, section 2.3). The first technique was used in *Hordeum* and *Brassica* seed lots. Longitudinal cuts were made through the seeds using a cryo-microtome (CM30505, Leica Biosystems, Nußloch, Germany). The second technique, only used on *Helianthus* seed lots, consisted of x-ray analysis to non-destructively record the morphological measurements. Images of the sectioned and x-ray seeds were taken under a stereoscope (Stemi SV 11, Carl Zeiss, Oberkochen, Germany) with an attached camera (AxioCam HRc 412-312, Carl Zeiss, Oberkochen, Germany).

To observe whether x-rays could have a deleterious effect on the seeds, 100 seeds of *H. annuus* (genotype B) were x-rayed and germinated at 25 ºC on germination paper with 7 mL of distilled water. This experiment was compared with 100 non-x-rayed seeds of the same seed lot germinated at the same conditions. The seeds were distributed in four replicates of 25 seeds each. There were no differences between them in rate nor in total germination (35.27 h and 35.31 h and 97.0 % and 98.0 % x-rayed and non-x-rayed respectively; $P > 0.05$ by t-test).

The seed measurements, in all seed lots, were the thickness of the seed coat or pericarp and the embryo length. The endosperm length was longitudinally measured in the endospermic seeds (*Hordeum* seed lots). Additionally, in the *Helianthus* seed lots the distance from radicle tip to the outside of the pericarp was measured. The mean and the coefficient of variation (CV) of all measures taken was calculated. The thickness of the seed coat or pericarp and the distance from radicle tip to the outside of the pericarp (in *Helianthus* seed lots) were divided by the mean embryo length (Weiss et al., 2013) to compare seed lots with different seed size.

3.2.5 Quantification of seed oil content

Intact seeds were used to measure the oil content using time domain nuclear magnetic resonance spectroscopy (TD-NMR, the minispec NMR analyser mq20, Bruker, Coventry, UK) (Borisjuk *et al.*, 2011). For each seed lot, the oil content was quantified in three replicates of seeds stored at 15 % RH (see Chapter 2, section 2.7). Data are expressed as % oil content (w/w).

3.2.6 Data analysis

Seed morphology measurements, mass and oil content were compared between seed lots using ANOVA followed by Tukey analysis to observe significant differences among seed lots. To compare two means (i.e. final water content or the rate of imbibition) the student's t-test (t-test) was used. The percentages were arcsine transformed prior to data analysis. Correlations between seed traits and the environment of the collection site of CWRs were plotted in a scatter matrix and linear regressions were performed.

3.3 Results

3.3.1 *Hordeum* species

The total seed germination of the *Hordeum* seed lots was above 80 % (Table 3.1). *H. pusillum* had the lowest germination percentage (83.9 %). However, seeds of *H. marinum*, two seed lots of *H. murinum* and the crops (commercial seed lot and the two treatments of *H. vulgare*), reached 100 % germination.

Seed mass in *Hordeum* CWRs ranged four-fold from means of 2.41 mg to 9.90 mg. *H. marinum* had the lowest seed mass and *H. bulbosum* from Greece had the heaviest seeds within the CWRs ($P < 0.05$, Table 3.1). However, the seeds of the crops were heavier than the CWRs seeds being at least 3 to 10-fold heavier than the CWRs seeds. The mean seed mass of the two crop treatments (control and drought) had similar values (Table 3.1), but the commercial seed lot had the heaviest seeds (50.58 mg, $P < 0.05$) and the least variability (CV = 0.1587) of the crops and CWRs.

Seed morphology was studied by measuring the embryo length, thickness of the seed coat and the endosperm length (see Chapter 2, Figure 2.1A). The CWRs seed lot with the largest embryo was *H. murinum* from Kyrgyzstan (1.651 mm, Table 3.1) and the embryo length of *H. pusillum* was the smallest (0.555 mm, $P < 0.05$) within the CWRs. Following the same trend as the seed mass, the thickness of the seed coat and the endosperm length were larger in the seed lot of *H. bulbosum* from Greece and smaller in *H. marinum* (Table 3.1). Although the crops had significantly heavier seeds than the CWRs, the thickness of the seed coat was similar between crops and CWRs seeds.

CWRs with heavier seeds had thicker seed coats ($P < 0.01$, Figure 3.1) than lighter seeds. The commercial seed lot, *H. vulgare*, had the thickest seed coat compared with the other crop genotypes (control and drought treatment, $P < 0.05$, Table 3.1). While the embryo length did not differ among the crop seed lots, the endosperm length of the commercial seed lot was significantly smaller than the endosperm of seeds from the crop genotypes (control and drought treatment, $P < 0.05$).

Seed oil content was quantified for all *Hordeum* seed lots. Seeds were very low in oil, with values of < 3 %.

The seed traits of all *Hordeum* seed lots (including both crop and CWRs) were compiled and subjected to linear regression analysis. There was a positive correlation between the endosperm and embryo length ($P < 0.05$, Figure 3.2 A). Moreover, the seed mass was also correlated with the embryo length ($P < 0.05$ Figure 3.2 B), but only if the crops were included in the analysis. Thus, seeds with large embryos possessed a large endosperm and were heavier than seeds with small embryos.

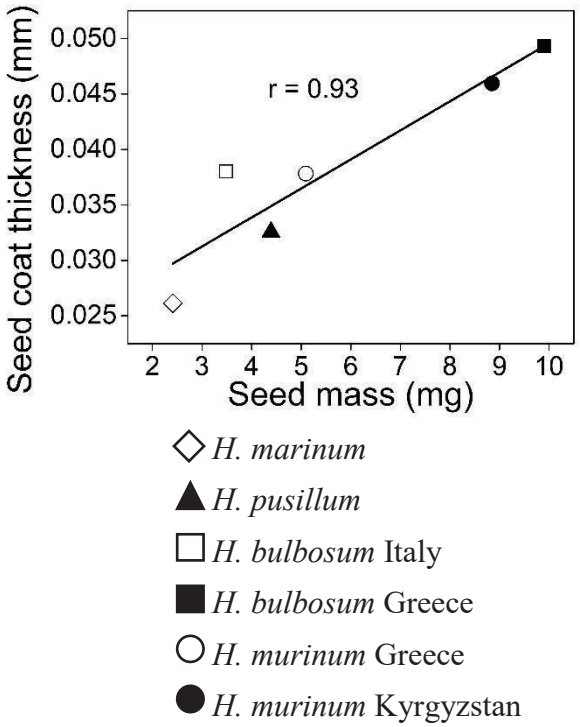


Figure 3.1 Significant correlation between the thickness of the seed coat and the seed mass of the six *Hordeum* CWRs ($P < 0.01$). DF = 4. Regression line in *Appendix* Table A3.3

Table 3.1 Seed characterisation of the *Hordeum* seed lots.Total seed germination percentage (G) is the mean of the highest germination percentage of three replicates for CWRs and four replicates for crops. Seed mass is the mean of individual weights measured on 100 seeds. Seed morphology measurements are the mean of 10 to 15 samples for embryo length, endosperm length and thickness of the seed coat. CV is the coefficient of variation. The mean values of the thickness of the seed coat were divided by the mean values of the embryo length. Different letters indicate the values were significantly different between CWRs and separately, between crops ($P < 0.05$).

CWRs	G (%)	Seed mass		Embryo length		Thickness of the seed coat		Endosperm length		Thickness /embryo
		mg	CV	mm	CV	mm	CV	mm	CV	
H. marinum Greece	100 a	2.41 b	0.2325	0.873 c	0.1927	0.026 d	0.1722	2.574 c	0.0970	0.030
H. bulbosum Italy	93.2 b	3.49 c	0.3959	1.064 ac	0.1261	0.038 b	0.1693	4.816 b	0.1424	0.036
H. pusillum USA	83.9 c	4.39 d	0.2961	0.555 d	0.1383	0.033 b	0.1841	2.339 c	0.1487	0.059
H. bulbosum Greece	93.3 b	9.90 a	0.2045	1.348 a	0.2346	0.049 a	0.2865	6.031 a	0.1011	0.037
H. murinum Greece	100 a	5.09 d	0.2279	1.433 ab	0.2775	0.038 bc	0.1845	4.515 b	0.1230	0.026
H. murinum Kyrgyzstan	100 a	8.85 e	0.3748	1.651 a	0.2277	0.046 ac	0.2329	5.037 b	0.1160	0.028
Crop *H. vulgare*										
Control treatment	100 a	25.98 f	0.3899	2.267 e	0.1257	0.038 e	0.1611	6.372 d	0.0793	0.017
Drought treatment	100 a	23.70 f	0.3560	2.076 e	0.1653	0.037 e	0.2300	6.442 d	0.0575	0.018
Commercial seed lot	100 a	50.58 g	0.1587	2.139 e	0.1306	0.051 f	0.3329	5.810 e	0.0724	0.024

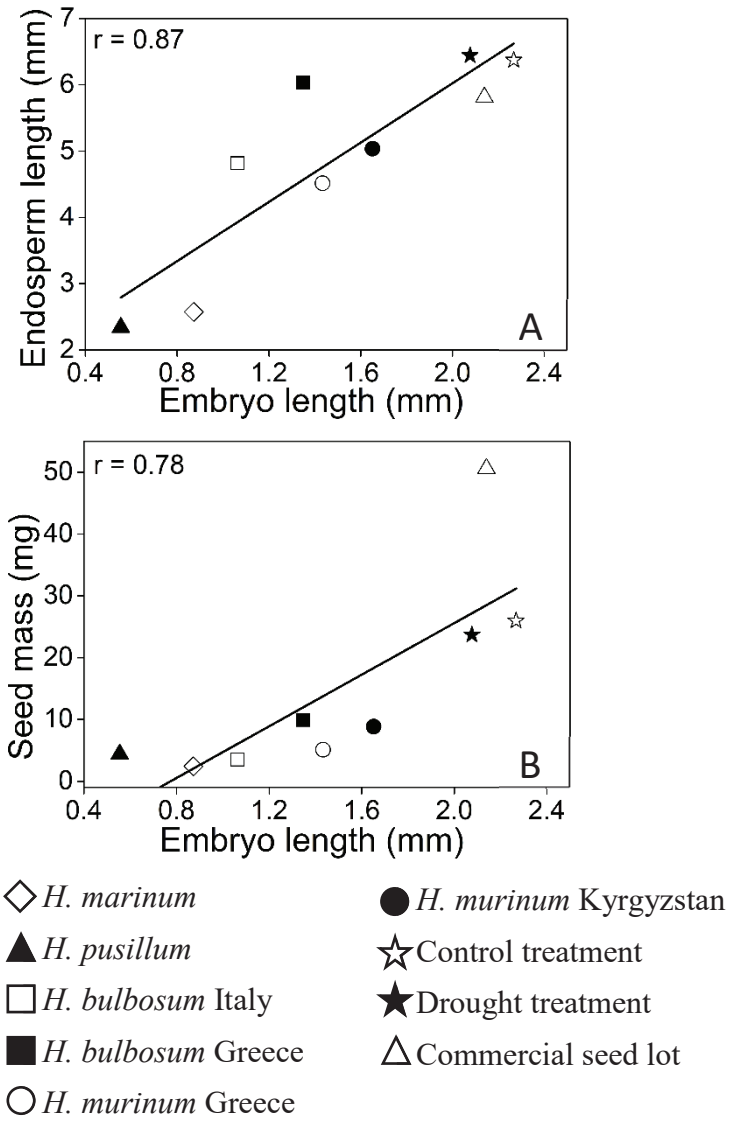


Figure 3.2 Correlations between seed endosperm and seed mass with embryo length of the *Hordeum* genus. Linear correlation between the endosperm length (A) and seed mass (B) with embryo length of nine *Hordeum* seed lots (six CWRs and three crops). Each symbol represents one seed lot. The regression lines were significant in both cases ($P < 0.05$, DF = 7). Regression lines are in *Appendix* Table A3.3

Using the data of the collection site environment described in Chapter 2, Table 2.3 for *Hordeum* CWRs, embryo length and the mean monthly precipitation was the only significant correlation ($P < 0.01$, Figure 3.3). Smaller embryos were found in seed lots from wetter environments.

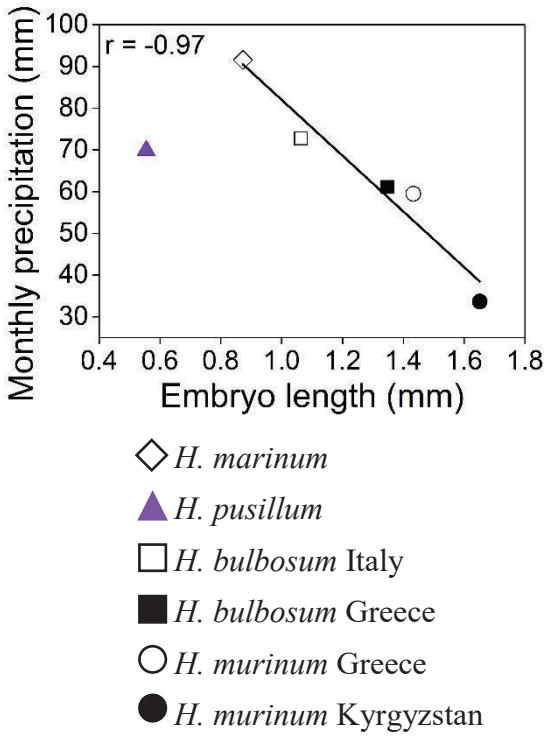


Figure 3.3 Significant negative correlation between the mean monthly precipitation of the environment of collection site and the embryo length of the *Hordeum* CWRs ($P < 0.01$, DF = 3). The correlation was significant if *H. pusillum* was not included in the analysis. Regression line is in *Appendix* Table A3.3

3.3.2 *Brassica* species

The seeds of all *Brassica* seed lots had high total seed germination. Seed germination of the CWRs ranged from 93.3 % (*B. rapa*) to 100 % (*B. rapa* subsp. *sylvestris*, Algeria, Table 3.2). The seeds of the commercial seed lot of *B. oleracea* and both research crop genotypes reached 99-100 % germination.

Seed mass varied among *Brassica* CWRs (Table 3.2). The mean seed mass of *B. rapa* from Switzerland was the greatest ($P < 0.05$) of the wild seed lots. In general, the crops had heavier seeds than the CWRs. Within the crops, the high vigour genotype AGSL101 had heavier seeds than the low vigour genotype A12DHd and the commercial seed lot of *B. oleracea* ($P < 0.05$, Table 3.2). The commercial seed lot was less variable in seed mass (CV = 0.1319) compared to the research crop genotypes and the CWRs. The drought treatment of A12DHd had a CV of seed mass almost half of the control treatment (Table 3.2).

In contrast to seed mass, the embryo length and the thickness of the seed coat (Chapter 2, Figure 2.1B) were less variable among the CWRs (Table 3.2).

Within the CWRs, the smallest embryo, 1.294 mm of *B. nigra*, was just 0.5 mm smaller than the largest (1.861 mm of *B. rapa* from France). The commercial seed lot had a slightly smaller embryo (1.828 mm) than *B. rapa* from France, and it was the smallest within the crops (Table 3.2). Similarly, the seed coat of the commercial seed lot was the thinnest compared to the other crop genotypes ($P < 0.05$). Seeds of the control treatment of the crop genotype AGSL101 (high vigour) has the largest embryo (2.163 mm), but the AGSL101 seeds of the drought treatment had the thickest seed coat ($P < 0.05$) compared to the other crop genotypes.

Table 3.2 Seed characterisation of the *Brassica* seed lots. Total seed germination percentage (G) is the mean of the highest germination percentage of three replicates for CWRs and four replicates for crops. Seed mass is the mean of 100 individual seed weights. Seed morphology data are the mean of 10 to 15 samples for embryo length and thickness of the seed coat. Mean oil content (%) was calculated using three replicates in crops and only one in CWRs. The coefficient of variation (CV) is the mean divided by the standard deviation. The mean values of the thickness of the seed coat were divided by the mean values of the embryo length. Different letters indicate the values were significantly different between CWRs and separately, between crops ($P < 0.05$).

CWRs	G (%)	Seed mass		Embryo length		Thickness seed coat		Thickness/ embryo	Oil content (%)
		mg	CV	mm	CV	mm	CV		
B. rapa (Switzerland)	93.3 a	3.50 a	0.2549	1.816 a	0.1368	0.036 a	0.1909	0.020	44.73
B. nigra (England)	94.7 a	1.33 b	0.3625	1.294 b	0.1164	0.027 cb	0.1688	0.021	34.26
B. rapa (France)	92.0 a	2.59 c	0.3671	1.861 a	0.0901	0.029 b	0.1872	0.015	42.00
B. rapa subsp. campestris (Turkey)	98.7 a	2.28 d	0.2533	1.718 a	0.1047	0.029 b	0.2023	0.017	35.34
B. rapa subsp. sylvestris (Morocco)	100 a	1.94 e	0.2287	1.652 a	0.1029	0.030 b	0.1711	0.018	48.46
B. tournefortii (Egypt)	97.3 a	1.40 b	0.1829	1.306 b	0.1150	0.025 c	0.2118	0.019	30.65
B. rapa subsp. sylvestris (Algeria)	100 a	1.67 f	0.2279	1.848 a	0.0809	0.029 b	0.2279	0.015	43.22
Crop *B. oleracea*									
A12DHd control treatment	100 a	3.58 g	0.3017	2.075 c	0.1347	0.045 d	0.1986	0.022	30.72
A12DHd drought treatment	99.0 a	3.71 g	0.1806	1.874 d	0.0548	0.049 e	0.1427	0.026	33.10
AGSL101 control treatment	100 a	4.55 h	0.2118	2.163 e	0.0844	0.042 d	0.1737	0.019	36.21
AGSL101 drought treatment	100 a	4.60 h	0.2449	2.004 cde	0.1063	0.055 f	0.1763	0.028	34.62
Commercial seed lot	100 a	3.50 g	0.1319	1.828 d	0.0932	0.030 g	0.2093	0.017	38.84

Positive correlations were found between seed mass and other seed measurements such as embryo length ($P < 0.001$) and thickness of the seed coat ($P < 0.001$) when data of both CWRs and crops were analysed together (Figure 3.4A). Additionally, the embryo length and the thickness of the seed coat were correlated to each other ($P < 0.05$, $r = 0.68$, Figure 3.4B).

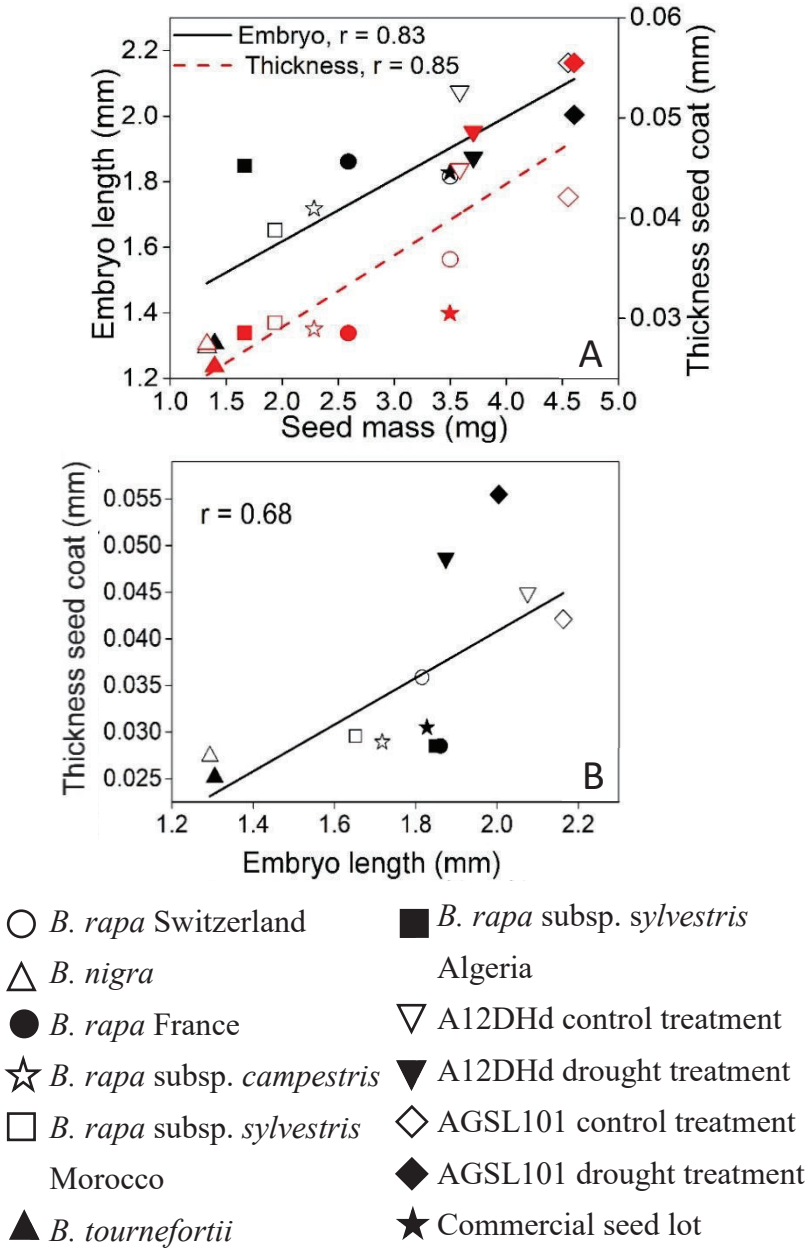


Figure 3.4 Correlations between physical seed traits of the *Brassica* genus. A) Significant correlation between the embryo length (solid line and black symbols) and seed coat thickness (dash line and red symbols) with seed mass ($P < 0.001$), and B) significant correlation between the thickness of the seed coat and the embryo length ($P < 0.05$) of seven *Brassica* CWRs and five seed lots of the crop *B. oleracea*. The regression lines were significant in both cases (DF = 10). Regression lines are in *Appendix* Table A3.3

Brassica seeds are typically high in oil. The oil content of CWRs varied from 48.46 % of *B. rapa* subsp. *sylvestris* (Morocco) down to 30.65 % of *B. tournefortii* (Table 3.2). The crop seed lots did not show a higher oil content than the wild seed lots. The commercial seed lot had the highest oil content of 38.84 % within the crop seed lots, but 10 % lower than the wild species *B. rapa* subsp. *sylvestris* (Morocco). There was not a consistent effect of the water treatment ("drought seed lots) on seed oil content in the research crop genotypes.

Linear correlations were studied between the seed traits of *Brassica* CWRs and the environment of the seed collection site described in Chapter 2, Table 2.3. However, the maternal environment of the seed collection site was not correlated with any seed trait presented in this chapter for *Brassica* CWRs (*Appendix* Table A3.4).

3.3.3 *Helianthus* species

Although non-scarified seeds of *H. argophyllus* reached 100 % germination, non-scarified seeds of the other four CWRs did not exceed 10 % germination (data not shown). Thus, to calculate the percentage seed germination of *Helianthus* CWRs it was first necessary to release dormancy. Scarification increased the germination of the four dormant seed lots with values of up to 40 % obtained when germinated with water (Table 3.3). In these seed lots, germination further improved ($P < 0.05$) when scarification was used in combination with GA_3 (Table 3.3), particularly at 1-10 mM GA_3 (Figure 3.5). Thus, for all further germination experiments, seed scarification was combined with 5 mM GA_3 to break dormancy in all *Helianthus* CWR seeds except *H. argophyllus* which seeds were germinated without any pre-treatment. Since not all seed lots reached 100 % germination, the non-germinated firm seeds were added to the total germination value to calculate their viability (Table 3.3).

Table 3.3 The effect of seed germination treatments on dormancy release in four *Helianthus* CWRs. Scarified seeds of the four dormant seed lots were germinated in water and with 5 mM gibberellic acid (GA_3). Germination percentage represent the mean (± standard deviation) of three replicates of 25 seeds. The stars (*) indicate significant differences between seed germination of scarified seeds in water and scarified seeds in GA_3 ($P < 0.05$). Viability is the number of germinated seeds plus non-germinated but firm seeds. Different letters denote significant differences ($P < 0.05$) between the seed lots.

CWRs	Germination (%)#		Viability (%)
	Water	5mM GA_3	
H. glaucophyllus	30.7 ± 6.8	86.2 ± 5.1*	96 a
H. angustifolius North Carolina	20.0 ± 5.7	78.9 ± 10.6*	89.3 a
H. angustifolius Texas	36.0 ± 13.6	89.5 ± 4.9*	92 a
H. pumilus	29.5 ± 15.5	81.9 ± 2.6*	96 a

temperatures used were: 15 °C (*H. pumilus*), 25 °C [*H. angustifolius* (from Texas) and *H. glaucophyllus*], and 30 °C [*H. angustifolius* (from North Carolina)].

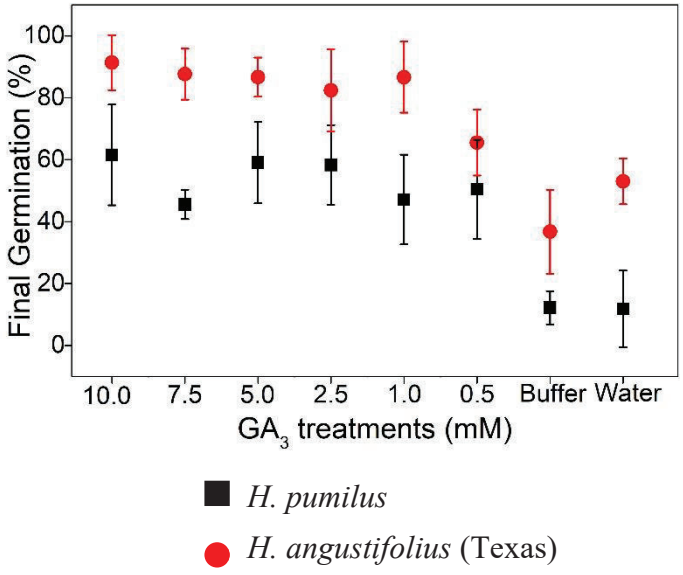


Figure 3.5 Scarified seeds of two CWRs of *Helianthus* perennial species (*H. pumilus* and *H. angustifolius* from Texas) germinated at six GA_3 concentrations (prepared in buffer) and two controls of buffer and water. There were no significant differences in total seed germination in GA treatments in *H. pumilus* or between 1.0 mM and 10.0 mM GA_3 ($P > 0.05$) in *H. angustifolius*.

The seed lots of Limagrain genotypes of *H. annuus* (sunflower) did not exhibit dormancy, however, physical damage was observed in the embryos. The seeds had lesions on the cotyledons, due to a fungal infection (Figure 3.6). The fungus was identified by the Université Pierre et Marie Curie, France, using PCR as *Rhizopus* genus, (*Appendix* Figure A3.1). To identify the proportion of infected and non-infected seeds for each Limagrain genotype and irrigation treatment, x-ray images were taken in 100 seeds of each seed lot (Figure 3.6). Most of the damaged cotyledons had small holes, visible on the x-ray, where the fungus had established. Some of these "spotted" seeds, assumed to be infected, were opened to confirm the fungal infection by visual inspection.

The seeds from Limagrain genotype B had the highest level of infection in both treatments, normal and stopped irrigation (Table 3.4). Despite the fungal infection, seed germination was above 80 % because the lesions were in the cotyledons and not the embryonic axis, thus, most of the seeds were able to germinate.

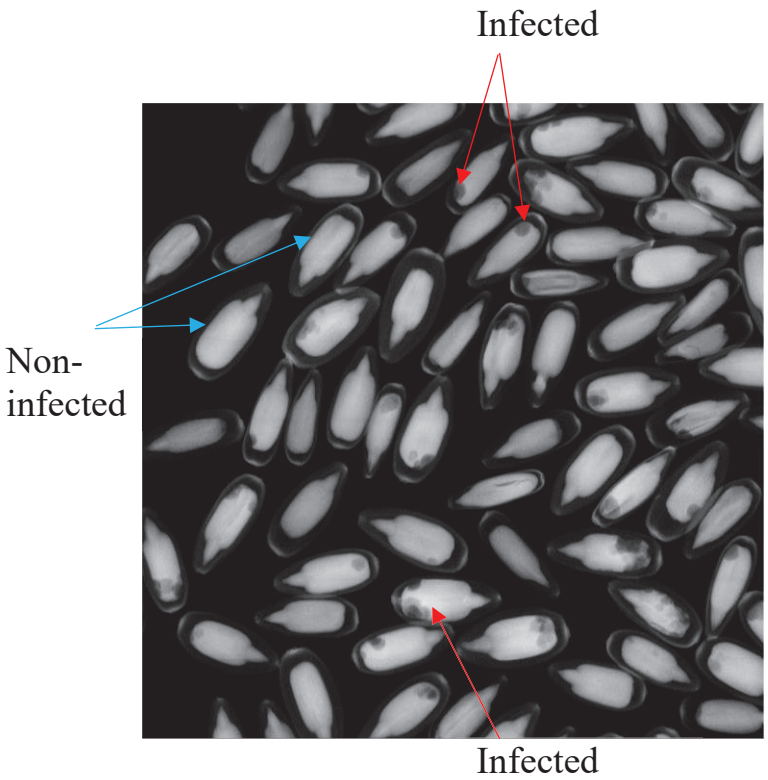


Figure 3.6 X-ray image of *Helianthus annuus* genotype B, normal irrigation with infected and non-infected seeds.

Table 3.4 Level of fungal infection of the Limagrain genotypes of *Helianthus annuus* based on the x-ray of 100 seeds. Seed germination percentage (G) represent the mean of three replicates of 25 seeds incubated at specified temperature (*Appendix* Table A3.1). There were no differences in G between the irrigation treatments within each crop genotype ($P < 0.05$).

Treatments	Genotype	Infected seeds (%)	G (%)
Normal irrigation	A	35	94.7 a
Stopped irrigation		30	92.2 a
Normal irrigation	B	57	88.8 a
Stopped irrigation		54	98.0 a
Normal irrigation	C	9	100.0 a
Stopped irrigation		15	96.1 a
Normal irrigation	D	29	97.3 a
Stopped irrigation		24	100.0 a
Normal irrigation	E	6	100.0 a
Stopped irrigation		16	100.0 a

After seed scarification and GA_3 treatment, *Helianthus* CWRs still tended to have lower germination percentage compared to the other two genera studied (*Brassica* and *Hordeum*), but it was still over 78 % (Table 3.5). *H. argophyllus* was the only wild species that reached 100 % germination, and was also the only CWR without seed dormancy. *H. angustifolius* from North Carolina showed the lowest germination of 78.9 %. In general, the crop genotypes had higher germination percentage than the CWRs, even when there was internal damage to the cotyledons. However, the lowest seed germination amongst the crops was measured for genotype B in the normal irrigation treatment (88.8 %, Table 3.5).

The seed mass differed between the *Helianthus* CWRs. The seed lot of *H. angustifolius* from North Carolina had the lowest seed mass (0.86 mg) while *H. pumilus* produced the heaviest seeds of the CWRs (5.68 mg, $P < 0.05$, Table 3.5). The crop genotypes produced seeds 10-fold heavier than the wild seed lots and all of them had lower CV values. However, the seed mass among the crops also differed, from 46.48 mg in the crop genotype C to 74.28 mg in crop genotype E (both stopped irrigation treatment, Table 3.5). The effect of stopped irrigation during seed filling on seed mass was not consistent within the Limagrain genotypes.

X-ray images were used to measure the embryo length, thickness of the pericarp and the distance from the radicle tip to the outside pericarp (Chapter 2, Figure 2.1 C). The embryo length of the CWRs differed from 1.035 mm (*H. glaucophyllus*) to 2.799 mm (*H. argophyllus*, Table 3.5). Furthermore, seeds from *H. argophyllus*, which had the largest embryo also had the thickest pericarp, 0.154 mm, almost three-fold greater than the other CWRs ($P < 0.05$). The distance from the radicle tip to the outside of the pericarp differed among CWRs from 0.216 mm to 0.601 mm (*H. pumilus* and *H. angustifolius* from Texas, respectively). The embryo lengths of most of the crops were between 8.235 and 9.421 mm with the exception of the commercial seed lot with a significantly smaller embryo length of 7.930 mm ($P < 0.05$, Table 3.5). However, the thickness of the pericarp and the distance from the radicle tip to pericarp were larger in the commercial seed lot (0.339 and 2.031 mm respectively, $P < 0.05$).

Table 3.5 Seed characterisation of the *Helianthus* seed lots. Total seed germination percentage (G) is the mean of three replicates for CWRs and four replicates for crops. Seed mass is the mean of individual weight of 100 seeds. The seed morphology data is the mean of 100 samples for embryo length, thickness of the pericarp and distance from radicle tip to the outside of the pericarp. The mean percentage of the oil content was calculated using three replicates for crops and only one for CWRs. The coefficient of variation (CV) is the mean divided by the standard deviation. The mean values of the thickness of the pericarp and the distance from radicle tip to the outside of the pericarp were divided by the mean values of the embryo length. Different letters indicate the values were significantly different between CWRs and between crops ($P < 0.05$).

CWRs	G (%)	Seed mass		Embryo length		Thickness pericarp		Distance from radicle to pericarp		Thickness/embryo	Distance/embryo	Oil content (%)
		mg	CV	mm	CV	mm	CV	mm	CV			
H. glaucophyllus (North Carolina)	86.2 ab	3.86 b	0.2759	1.035 d	0.1128	0.063 b	0.1695	0.317 b	0.1910	0.061	0.307	32.65
H. angustifolius (North Carolina)	78.9 b	0.86 d	0.2344	1.385 b	0.1207	0.059 b	0.2823	0.403 c	0.1997	0.043	0.291	31.21
H. angustifolius (Texas)	89.5 ab	1.13 d	0.2543	2.083 c	0.1374	0.062 b	0.1296	0.601 a	0.2174	0.030	0.289	34.48
H. argophyllus (Texas)	100.0 a	4.63 c	0.2095	2.799 a	0.1037	0.154 a	0.2850	0.526 a	0.1337	0.055	0.188	28.21
H. pumilus (Colorado)	81.9 ab	5.68 a	0.1995	1.229 e	0.1317	0.069 b	0.2831	0.216 d	0.2439	0.056	0.175	24.22
Crop *H. annuus*												
A normal irrigation	94.7 ab	64.15 e	0.1581	8.745 g	0.0800	0.283 cd	0.2110	1.247 df	0.1507	0.032	0.143	43.15
A stopped irrigation	92.2 ab	65.78 e	0.1597	9.421 f	0.0662	0.300 cd	0.2367	1.374 gf	0.1545	0.032	0.146	40.37
B normal irrigation	88.8 b	48.97 fg	0.1308	8.235 hi	0.0522	0.313 de	0.1705	1.232 def	0.1610	0.038	0.150	38.65
B stopped irrigation	98.0 ab	54.39 fh	0.1290	8.545 gh	0.0780	0.285 cd	0.2333	1.252 df	0.1390	0.033	0.146	41.90
C normal irrigation	100.0 a	63.15 ei	0.1419	8.562 gh	0.0818	0.290 cd	0.2166	1.098 de	0.1386	0.034	0.128	47.37
C stopped irrigation	96.1 ab	46.48 g	0.1084	8.325 h	0.0573	0.256 c	0.1757	1.041 de	0.1496	0.031	0.125	46.45
D normal irrigation	97.3 ab	67.89 ej	0.1570	9.271 f	0.0599	0.305 ce	0.1881	1.251 dgf	0.1632	0.033	0.135	46.30
D stopped irrigation	100.0 a	57.57 ik	0.1456	8.817 g	0.0723	0.282 ce	0.1949	1.296 gf	0.1690	0.032	0.147	42.78
E normal irrigation	100.0 a	68.89 ej	0.1606	8.504 gh	0.0529	0.266 ce	0.1686	1.135 d	0.1359	0.031	0.134	45.72
E stopped irrigation	100.0 a	74.28 j	0.0957	8.722 g	0.0444	0.279 ce	0.1750	1.169 df	0.1798	0.032	0.134	44.30
Commercial seed lot	99.0 a	57.48 ik	0.1564	7.930 i	0.0776	0.339 cd	0.2759	2.031 h	0.1578	0.043	0.256	32.18

Sunflower, an important oilseed crop, showed the highest seed oil content compared to the other two genera (*Brassica* and *Hordeum*). The oil content of the CWRs differed among them. *H. pumilus* had the lowest oil content (24.22 %), while seeds of *H. angustifolius* from Texas showed the highest oil content among CWRs (34.48 %, Table 3.5). All crop genotypes possessed higher oil contents than the CWRs, but the percentages were similar among the crops. Seeds from crop genotype B, normal irrigation, showed the lowest oil content (38.65 %) while the highest oil content was measured in seeds of crop genotype C, normal irrigation (47.37 %, Table 3.5). Therefore, the effect of stopped irrigation on the oil content was not consistent among Limagrain genotypes.

Seed morphology data and seed mass from CWRs were analysed separately from crops due to scale differences (Figure 3.7). There were no correlations between seed traits within the CWRs. However, the oil content of the crop genotypes was negatively correlated with the distance from radicle tip to the outside of the pericarp ($P < 0.001$, $r = -0.89$, Figure 3.8).

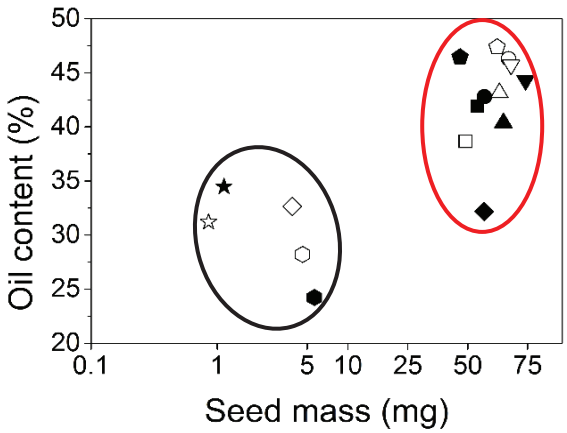


Figure 3.7 Example of the differences in the scale of seed mass in *Helianthus* CWRs and crops. The oil content is plotted against a log scale of seed mass. The data points enclosed in the black circle are CWRs and the points enclosed in the red circle are crop seed lots.

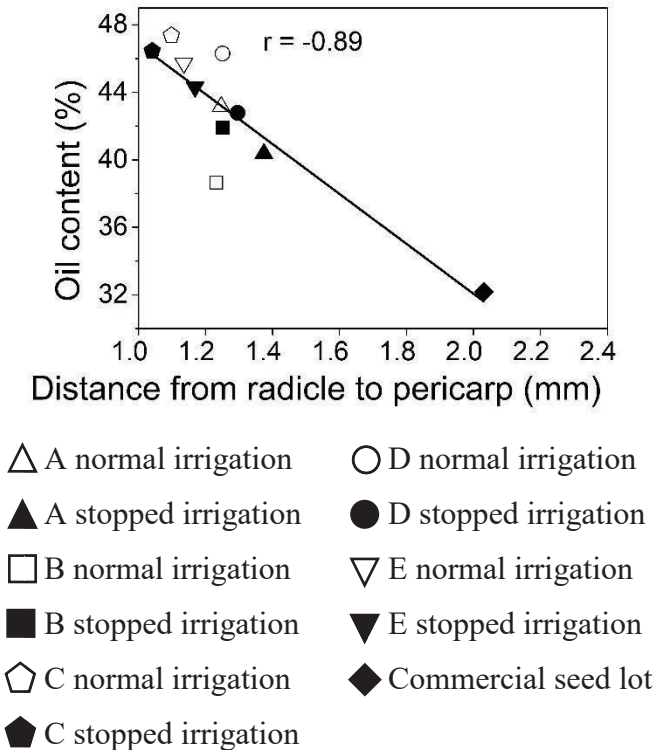


Figure 3.8 Significant correlation between the oil content and the distance from radicle tip to the outside of the pericarp of six crop genotypes of *Helianthus annuus* ($P < 0.001$). Mean values of two treatments of the crop genotypes of *H. annuus* (genotypes A to E) were included, normal irrigation and stopped irrigation treatment. Each symbol is the mean of three replicates for oil content and 15 seeds for distance from radicle tip to the outside of the pericarp (DF = 9). Regression line is in *Appendix* Table A3.3

Seed morphology (embryo length, thickness of the pericarp and distance from radicle tip to the outside of the pericarp), seed mass and oil content of the *H. annuus* crop genotypes also differed among them. However, the effect of the stopped irrigation treatment during the seed filling stage was not consistent.

Using data from the environment of the seed collection site described in Chapter 2 Table 2.3 of *Helianthus* CWRs, a positive correlation was found between embryo length and the annual mean minimum and mean temperature (Figure 3.9A, $P < 0.05$, $r = 0.89$ in both cases). In addition, the distance from the radicle tip to the outside of the pericarp was also positively correlated to the annual mean minimum, mean and maximum

temperature (P < 0.05, r = 0.92, r = 0.91 and r = 0.92 respectively, Figure 3.9B).

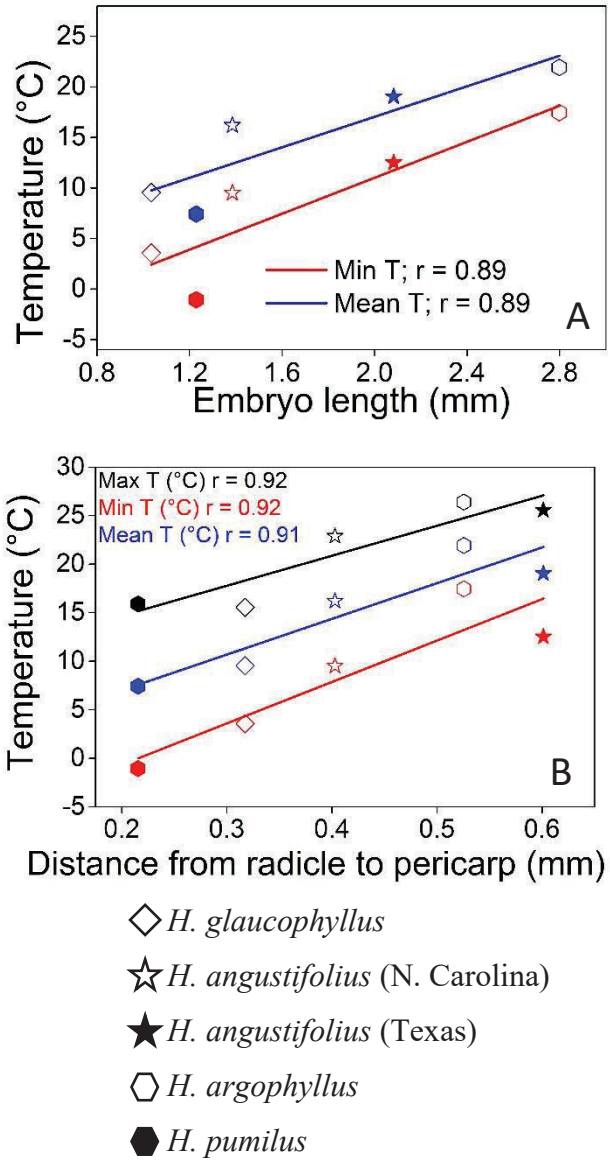


Figure 3.9 Correlations between the physical seed traits and the maternal environment of *Helianthus* CWRs. A) Significant correlations between the annual mean temperature (maximum temperature in black, minimum temperature in red and mean temperature in blue) of the seed collection site and the embryo length and B) the distance from radicle tip to the outside of the pericarp of CWRs seeds of *Helianthus*. Each symbol represents one population of *Helianthus* CWRs. The linear regressions were significant (P < 0.05, DF = 3 in both). Regression lines are in *Appendix* Table A3.3

3.4 Discussion

Variation in mass was linked to several seed traits

Due to domestication and human selection over the last centuries, crops have been developed to have heavier and larger seeds compared to their respective CWRs. Natural selection has left high genetic diversity across the wild species of the world (Hajjar & Hodgkin, 2007) therefore, more diversity is expected across CWRs than within the crops of the same genera. Seed mass of the CWRs of the three genera was the most diverse seed trait compared to their respective crop seed lots. Differences in seed mass between CWRs and crops were reflected in embryo length for the *Hordeum* and *Brassica* genus. In *Hordeum*, longer endosperms were found in seeds with longer embryos. López-Castañeda *et al.* (1996) found that longer embryos were correlated with early emergence of barley seeds. The hypothesis that longer embryos have faster seed germination is explored in Chapter 4.

In general, species with larger seeds produce larger seedlings (Westoby *et al.*, 2002) or can emerge from greater depths (Bond *et al.,* 1999) within the soil probably because they have larger storage reserves. Different to *Hordeum* seeds, *Brassica* seeds are not endospermic and its storage tissue is the embryo (in particular, the cotyledons). Seed size and seed mass have been previously related to the presence of storage tissue (Hodgson & Mackey, 1986; Moles & Westoby, 2004b; Bewley *et al.*, 2013). In this case, heavier seeds of *Helianthus* and *Hordeum* did not have longer embryos nor endosperms, which are the main storage tissues, respectively. This chapter considers embryo and endosperm length, however using other measurement such as width or volume of the seeds could correlate with seed mass.

In some cases where greater seed mass is not related to larger storage tissue, the increase in mass could be related to a thicker seed coat (Lacey *et al.*, 1997). Such correlation was true for the seed lots of the *Brassica* genus and the *Hordeum* CWRs (Figure 3.1 and 3.4A) studied in this chapter. The lack of correlation between the thickness of the pericarp and seed mass in the *Helianthus* genus could be due to the relatively consistent thickness of the pericarp within crop genotypes and within CWRs (Table 3.5).

In *Helianthus* seeds, selection to obtain higher seed mass was more obvious in the commercial seed lot where the seeds were heavier (Table 3.5) than in seeds of the Limagrain genotypes (control and drought treatments). The commercial seed lot of *Helianthus annuus* was provided by a seed company whose primary purpose is to produce homogeneous seeds in size, mass and germination rate. Moreover, the coefficient of variability (CV) of seed mass in the commercial seed lots (provided by B&T World Seeds company) of *Hordeum* and *Brassica* was lower than the other crop seed lots of the same species (provided by IPK and University of Warwick respectively). Seed companies address the demands of the agricultural industry to have standard seed sizes and masses to optimise sowing techniques and maximise yield by screening the seeds for their size (length and width) mass and shape (e.g., smallest seed sizes are removed as non-viable), thus obtaining more homogeneous seed lots.

The effect of high vigour alleles in the *B. oleracea* genotype AGSL101 was reflected in heavier seeds compared to the low vigour crop genotype (A12DHd). The Limagrain genotypes of *Helianthus* have differences in their seed oil content and composition. These differences did not have a consistent effect on seed mass, and thus, of the traits measured in this chapter, the seed mass of *Helianthus* is less predictable than the other two genera and likely influenced by multiple traits in combination with the environment, especially since *Helianthus* Limagrain genotypes were grown in the field.

In general, the effect of domestication and breeding on seed mass have been reflected in heavier seeds and more homogeneity in the crop seed lots compared to the CWRs. This effect may have an impact on seed germination and will be explored in the following chapters for each genus.

Variation in oil content of the oilseed genera

Crops of *Brassica* have heavier seeds than their CWRs, but oil content was not higher on the crop seeds. This could be because crop types of *B. oleracea*, which are known as cabbage, kale and broccoli, are vegetables marketed for their vegetative yield rather than seed, and thus have not been bred for high seed oil content. The drought treatment and the high vigour alleles did not have a significant effect on seed oil content.

On the other hand, the crop seed lots of *H. annuus* had higher oil contents than their CWRs. In this case, *H. annuus* has been bred to produce a high quantity of oil in their seeds as this is the main component of yield. The oil content and the distance from radicle tip to the outside of the pericarp were negatively correlated. However, the distance between the radicle and the outside of the pericarp is an air gap, thus a larger distance would relate to less storage tissue where the oil is stored.

Effect of the environment of seed collection site on seed traits across genera

The compiled seed characterisation data in the CWRs of the *Hordeum, Brassica* and *Helianthus* genera showed correlations with the seed collection site environment. Previously, it has been hypothesised that variation in embryo length is related to differences in the habitat of the species (Fenner & Thompson, 2004). In the European CWRs of *Hordeum* used in this chapter, seeds whose maternal environment was dry had longer embryos than from wetter environments. This finding is consistent with Vandelook *et al.* (2012), who found that seeds of *Apiaceae* species with long embryos were from environments with a low amount of precipitation. Additionally, they found this correlation more advantageous (i.e. faster germination) under non-optimal conditions than slow germination linked to seeds with small embryos. Further studies with more species from the American taxa should provide a more complete characterisation of the *Hordeum* genus. Nonetheless, the question of whether there is an advantage of long embryos over short embryos on seed germination rate is explored and discussed in the next chapters separately for each genus (Chapter 4 for *Hordeum,* Chapter 5 for *Brassica* and Chapter 6 for *Helianthus*).

Several experiments in the field have suggested that seed mass is a plastic trait that varies with differences in the maternal environment (Leishman *et al.*, 1995; Wulff, 1995; Leishman *et al.*, 2000). In particular, precipitation changes affected seed mass in several species such as *B. campestris* (Sinniah *et al.*, 1998), annual species (e.g. *Glycine* genus) from a Mediterranean climate (Murray *et al.*, 2004) and two wild species of barley and oat (Volis, 2012). In this chapter, the seed mass of the CWRs

was not correlated with any factor in the maternal environment (annual mean temperature and mean monthly precipitation). Nonetheless, the relationship between seed mass and the precipitation of the month of germination of the seed collection site is explored in subsequent chapters, where seed germination traits are described and month of germination is defined.

In the case of *Helianthus* CWRs, precipitation was not the main environmental factor affecting seed traits. Seeds that were produced at higher temperatures had longer distances between the radicle tip and the outside of the pericarp than those produced at lower temperatures. However, this may not be a disadvantage for the seeds in terms of germination. Seeds may have the capacity to germinate faster (i.e. extend the radicle) because they will germinate under warmer temperatures (Fenner, 1991; Wulff, 1995). Moreover, Weiss *et al.* (2013) found that seeds of *H. annuus* crop had shorter distances between the radicle tip and the outside of the pericarp, when divided by the embryo length, than the wild seeds. This was generally the case for the *Helianthus* genus. Weiss et al., (2013) suggested that crop seeds will emerge faster than the wild seeds due to shorter distances. These hypotheses are investigated in Chapter 6.

The effect of water-limiting treatments on the seed traits of *Brassica* and *Hordeum* crop genotypes (drought) and *Helianthus* Limagrain genotypes (stopped irrigation) was not consistent. For example, the drought treatment in *H. vulgare* did not have any effect on the seed morphology, seed mass or total germination percentage. This lack of differences between control and drought treatments could be because the mother plant was under limited water for a short period of time (the seed filling until seed harvest). Farahani *et al.* (2010) found that ceasing irrigation of the mother plant during a shorter period (from the beginning of flowering to initiation of seed filling) had less impact on seed mass and mean time to germination (MTG) than ceasing irrigation applied for a longer period (from the beginning of flowering to the end of seed physiological maturity). Therefore, the effect of limiting water to the mother plant is pronounced when the treatment is applied over longer time (Farahani *et al.*, 2010), in addition to the importance of the plant stage

(e.g., flowering time, seed filling or seed maturation) when the treatment is applied. Moreover, extreme weather events, such as drought, have implications for understanding the likely impacts of climate change on seed functional traits.

The water treatment in the *B. oleracea* crop did not affect the seed mass since there were no significant differences between treatments in either genotype A12DHd or AGSL101. In contrast, the embryo length and the thickness of the seed coat were affected by the water treatment applied in both crop research genotypes. Soybean plants under a different type of stress (reduced minerals and cytokinin) also produced seeds with thicker seed coats (Nooden *et al.*, 1985). The effect of different environmental conditions on the seed coat has been studied before in relation to light in Fabaceae (Gutterman, 1978) and altitude in Chenopodiaceae (Dorne, 1981). Although the morphology (structure and composition) of the seed coat is an important seed characteristic of *Brassica* used to classify species in the phylogeny (Zeng *et al.*, 2004; Kasem *et al.*, 2011), to my knowledge, there is no information in the literature about the influence of the environment on the seed coat thickness of *B. oleracea*.

The differentiating characteristics of the *Helianthus* Limagrain genotypes were the oil composition and the earliness of flowering (see Chapter 2, Table 2.5). The stopped irrigation treatment was performed for the same period of time in all Limagrain genotypes (end of July to the beginning of September). The lack of consistency in the water treatment effect on seed traits may have resulted from the different genetic composition or the different flowering times and therefore timing of the seed filling of the Limagrain genotypes. Nonetheless, these results could suggest plasticity of physical seed traits over a range of conditions in the *Helianthus* crop genotypes.

Seed dormancy of *Helianthus* CWRs

Seed coat and pericarp are seed coverings that in some species have been shown to mechanically restrain the radicle, prevent water from reaching the embryo, or block the escape of inhibitors from the embryo (Baskin & Baskin, 1998; Finch-Savage & Leubner-Metzger, 2006; Gosling, 2006; Rathjen *et al.*, 2009). In my study, when the seed

morphological measurements were expressed as a proportion of the embryo length, the seeds of *Helianthus* CWRs showed longer distances from the radicle tip to the outside of the pericarp, and thicker pericarps, than crop seed lots. Although the seeds of *Helianthus* CWRs did not have impermeable tissues (*Appendix* Table A3.2), the pericarp did serve as a partial physical barrier (Esashi & Leopold, 1968), as radicle emergence improved with scarification in the perennial seed lots. Relatively thicker seed covering structures can serve as protection to predation and is associated with increased persistence and decreased mortality in the soil seed bank (Fenner, 1983; Blate *et al.*, 1998; Gardarin *et al.*, 2010; Schutte *et al.*, 2014). In the experiments reported it is likely that the pericarp was not the only cause of seed germination impedance since seed scarification was not enough to reach high seed germination. Thus, a combination of GA_3 and scarification was needed to break seed dormancy in higher percentage on *Helianthus* CWRs.

Previously, Chandler and Jan (1985) experimented with several techniques to break dormancy in seeds of wild *Helianthus* species. They concluded that removal of the pericarp and soaking the seeds with GA was the best technique to obtain higher germination percentages for most of the wild *Helianthus* species. However, this operation is difficult to apply to small seeds such as *H. angustifolius*, often resulting in some damage to the embryo. To circumvent this problem, a simpler approach was adopted of partial removal of the pericarp at the embryonic axis end. However, this intervention alone was insufficient to ensure high levels of germination. Most of the wild species required scarification and GA_3 treatment, indicative of the presence of deep physiological dormancy (defined by Baskin and Baskin, 1998, Finch-Savage and Leubner-Metzger, 2006). Based on the viability estimated from firm (i.e. excluding empty seeds) and non-infected seeds on *Helianthus* CWRs, there was between 3 to 14 % of viable seeds where dormancy was not removed (Table 3.3). Thus, the technique to overcome dormancy used in this study on perennial *Helianthus* CWRs succeeded in a minimum of 85 % up to 97 % of the viable seeds. *H. argophyllus* was the only wild species studied without dormancy. Chandler and Jan (1985) scarified seeds of *H. argophyllus* and

obtained 100 % germination without GA, however, they did not show the capacity for germination without scarification. In the present study, *H. argophyllus* seeds were able to germinate (reaching 100 %) without scarification or GA. In the phylogeny described by Timme *et al.* (2007) *H. argophyllus* and *H. annuus* are from the same clade or section (*Helianthus*) and are annual species. Thus, it is reasonable to assume that *H. argophyllus* and *H. annuus* would have similar characteristics, such as the absence of seed dormancy.

In contrast to results described by Debeaujon et al. (2000); Baskin & Baskin (2004) and Maass (2006), the dormancy of the CWRs of *Helianthus* was not due solely to a thick pericarp since the dormant seed lots had the thinnest pericarps. In other studies where the thickness of the pericarp was related to seed dormancy, that relationship existed because the seeds were impermeable to water (Nooden *et al.*, 1985; Schutte *et al.*, 2014). In any case in this work *Helianthus* CWRs did not have impermeable pericarps since the seeds imbibed water without scarification (*Appendix* Table A3.2).

3.4.1 Conclusions

CWRs were compared to crops to observe the effect of breeding and domestication on the three genera, one of the main effects of which is greater seed mass. The environment of the collection site of the CWRs had a significant impact on seed traits. Mean monthly precipitation was the main factor affecting embryo length and seed mass for seed lots in the *Hordeum* and *Brassica* genus respectively. Annual mean temperature was the main environmental factor that had an impact on the *Helianthus* CWRs as it was related to the distance from radicle tip to the outside of the pericarp. On the other hand, the impact of water stress on the mother plant (irrigation treatments for *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus*) was not consistent among the crops.

In the following chapters, seed germination in the seed lots described here is explored under a wide range of temperatures and water potentials. The influence of the seed traits discussed in this chapter are further analysed in relation to seed germination. Additionally, the impact of the

fungal infection on seed germination and normal seedling growth of *Helianthus annuus* is reported in Chapter 6.

4 CHAPTER 4: QUANTIFYING GERMINATION PERFORMANCE IN CROP WILD RELATIVES OF THE *HORDEUM* GENUS AND THE CROP *H. VULGARE*.

4.1 Introduction

The *Hordeum* genus belongs to the family Poaceae. The family includes important cereal crops such as rice, wheat and barley. As monocotyledons, the seeds of *Hordeum* species have a single cotyledon. The seed also has an endosperm and is surrounded by a seed coat. The endosperm is a storage tissue that provides reserves to the embryo during the germination process. The endosperm size is correlated with the seed size (Leishman *et al.*, 2000; Bewley *et al.*, 2013), so that larger seeds should have higher quantities of reserves. Embryo size has been studied in *Hordeum* seeds (López-Castañeda *et al.*, 1996) in relation to seedling vigour and it was concluded that larger embryos produced more vigorous seedlings, i.e. more advantageous than smaller embryos.

Climatic events such as extreme temperatures and rainfall patterns impact on the duration of the growing season, and drought is the main cause of yield losses in barley (Newton *et al.*, 2011b). Cereals are particularly susceptible to the combination of drought and high temperatures during grain filling, which can have a negative impact on yield (Samarah, 2005; Barnabás *et al.*, 2008). Plant vigour is also reduced by these conditions resulting in the development of smaller seeds (Szira *et al.*, 2008) and consequently reduced seed mass (Savin & Nicolas, 1996; Samarah, 2005). Previous investigations in wild species of *H. spontaneum* have studied how seed germination and seedling development are influenced by fluctuations in temperature (Zhang & Gutterman, 2003) and the season in which precipitation occurs (Gutterman & Gozlan, 1998). Salinity and drought in the seedbed can affect seed germination, by limiting the water absorption of the seed, and thus reducing seed germination percentage and rate (Mer et al., 2000, Farahani et al., 2010). The diversity and adaptability of the CWRs could be of major importance to the perpetuation of the wild populations. In addition, the diversity of the

Hordeum CWRs could be used to improve barley as a crop under climate change. For example, Nevo and Chen (2010) found that *H. spontaneum* has traits associated with seedling drought tolerance and *H. marinum* has been described as a salt tolerant species (Colmer *et al.*, 2006; Alamri *et al.*, 2013). Thus, wild *Hordeum* species have become a desirable genetic source due to their abiotic tolerance (Newton *et al.*, 2011a).

The seed germination response to the environment can be described by population-based threshold modelling approaches such as thermal (temperature) and hydro (water potential) time (García-Huidobro et al., 1982, Bradford, 1995; Chapters 1 and 2). This modelling approaches include the response of the whole seed population and as such provide a powerful tool to analyse the response of seed germination to environmental change. The literature contains little information on the thermal and hydro parameters of species in the *Hordeum* genus. Dürr et al. (2015) compiled data of thermal time (θ_T), hydro time (θ_H), base temperature (T_b) and base water potential (Ψ_b) values of 243 species, of which just one species was from the *Hordeum* genus. Another study estimated the Ψ_b of barley (Ψ_b = -1.75 MPa) (Zhang *et al.*, 2010). Furthermore, seed population of *H. comosum* was analysed by Gundel *et al.* (2012) to calculate the θ_T (768.74 ºCh), T_b (-1.1 ºC), θ_H (26 MPah) and Ψ_b (-0.99 MPa).

In this study, six seed lots (four species) of *Hordeum* CWRs (Chapter 2 Table 2.3) were selected from different locations to cover a broad representation of *Hordeum* growth habitats. Following the phylogeny published by Petersen and Seberg (2003) that described four clades in *Hordeum*, the CWRs and crop selected are from two clades: the American taxa (*Critesion* clade that includes *H. pusillum*) and the Eurasian taxa (*Hordeum* clade that includes *H. marinum, H. murinum, H. bulbosum* and *H. vulgare*), all of which are annual species (Blattner, 2004). *H. vulgare* seeds were obtained from IPK and a commercial company (see Chapter 2, Table 2.4). Seeds from IPK were produced in two experimental water treatments, control and drought (see Chapter 2, section 2.2.3). In addition, to the seeds from these two treatments, seeds of a commercial seed lot are also used and all three seed lots will be referred as crops in this study.

The month of germination for the CWRs was estimated using climate data for the environment of their collection site described in Chapter 2 Table 2.3. Moisture is arguably the most important factor for seed germination for most of the species because the germination process usually begins with imbibition (Bewley, 1997). However, the seeds of *Hordeum* CWRs need a period of dry after-ripening to break dormancy in nature (Baskin & Baskin, 1998; Gutterman & Gozlan, 1998; Bewley *et al.*, 2013), before germination can commence. The seeds of *Hordeum* CWRs were collected at maturity before or at the beginning of summer (information provided by the collectors and storage database, *Appendix* Figure A4.1). Thus, the seeds were ready to germinate when significant rain falls after summer. Therefore, the predicted month of germination for the CWR seed lots is based on the following assumptions (1) the minimum rainfall required for germination is a monthly mean of 15 mm (Freas & Kemp, 1983; Gutterman, 1993, 2000a) and (2) the temperature exceeded T_b (*Appendix* Figure A4.1).

The objective of this chapter is to characterise seed germination and the conversion into normal seedlings under different temperatures and water potentials (see Chapter 2, Table 2.6). On rice seeds, cell division on the radicle tip was stopped at 40 ºC (Yoshida, 1981). Thus, the hypothesis presented here is that high temperatures may have deleterious consequences on normal seedlings. To do this, seeds were exposed to a range of germination environments and the data collected were used to analyse germination behaviour and summarise germination functional traits. Thus, θ_T, θ_H, hydrothermal time (θ_{TH}), T_b, ceiling temperature (T_c) and Ψ_b were calculated in *Hordeum* CWRs and the crop *H. vulgare* for comparison. The correlations found between seed functional traits are discussed. Additionally, the effect of the water treatment on seed germination of *H. vulgare* was determined. It was hypothesised that the seeds from the drought treatment of *H. vulgare* have lower Ψ_b than those of the control treatment. Furthermore, relationships between seed morphology and seed mass (described in Chapter 3) and seed germination traits were explored. The hypothesis presented regarding Chapter 3 is that larger and heavier seeds germinate faster, i.e. shorter θ_T and θ_H, than

smaller and lighter seeds. Finally, conclusions were drawn on the impact of the environment of seed collection site (see Chapter 2, Table 2.3) on seed germination traits in the CWRs.

The statistical analyses performed to compare seed germination traits between CWRs and crops were analysis of variance (ANOVA), Tukey test and t-tests. The percentages were previously arcsine transformed. In addition, correlations between the environmental factors and the seed germination traits of the CWRs, θ_T, θ_H, T_b and Ψ_b were tested with scatter matrix and linear regressions in Origin 9.0 software (OriginLab Corporation, 2013).

4.2 Results

4.2.1 Germination traits of seeds from the *Hordeum* genus

Thermal time: All T_b values were lower than 3 °C (Table 4.1). The lowest T_b, within the CWRs, was -1.8 °C in *H. marinum* while the highest was 2.6 °C in *H. murinum* from Greece (Figure 4.1 A, C and E). However, the commercial seed lot, *H. vulgare*, had the lowest T_b compared to all of them (-2.3 °C; Table 4.1). Thermal time (θ_T) was similar among the CWRs, except *H. pusillum* that had the longest θ_T (2634.7 °Ch) (Table 4.1). In general, the crop seeds germinated faster than seeds of the CWRs (i.e. θ_T values were lower). Seeds of *H. vulgare* from the control treatment had the fastest germination compared with the other two *H. vulgare* seed lots ($P < 0.05$) (Table 4.1).

The supra-optimal thermal (θ_{Tsupra}) time was only calculated in two CWRs. The ceiling temperature (T_c) was 25 and 33.5 °C for *H. pusillum* and *H. murinum* from Greece respectively (Table 4.1). Both show a lower T_c than the crops and longer θ_{Tsupra}; 600 °Ch for *H. pusillum* and 3997 °Ch for *H. murinum* Greece. In the supra-optimal range of temperatures (from 30 °C to 40 °C), the water treatments (control and drought) of *H. vulgare* did not influence the θ_{Tsupra} values nor T_c (Figure 4.1 G). However, the commercial seed lot differed from seeds of the treatments (Table 4.1) and had a wider thermal range for germination (Figure 4.1 G). In general, seed

lots with higher T_c germinated slower (longer θ_T) than seed lots with lower T_c.

Hydro time: The lowest Ψ_b was found in *H. marinum* (Ψ_b = -1.75 MPa) (Figure 4.1 B and Table 4.1). *H. murinum* from Greece had the highest Ψ_b, -0.81 MPa (Figure 4.1 F). The Ψ_b of crop seeds was within the range of CWRs (Table 4.2). Differences between the drought and the control treatments in *H. vulgare* were found. While seeds of the control treatment and the commercial seed lot had the same Ψ_b, seeds of the drought treatment had a lower Ψ_b value (Figure 4.1 H). In general, the crop seeds had lower θ_H values than those of the CWRs. *H. pusillum* was the species having seeds with the longest θ_H (174.3 MPah). In contrast, seeds of the control treatment of *H. vulgare* had the shortest θ_H (33.5 MPah).

Hydrothermal time: As expected, seeds of the species described with the shortest θ_T and θ_H also had the shortest hydrothermal time (θ_{HT}), and vice versa for the species described with the longest θ_T, θ_H and θ_{HT}. Thus, seeds of the crops had lower θ_{HT} values compared with those of the CWRs (Table 4.1).

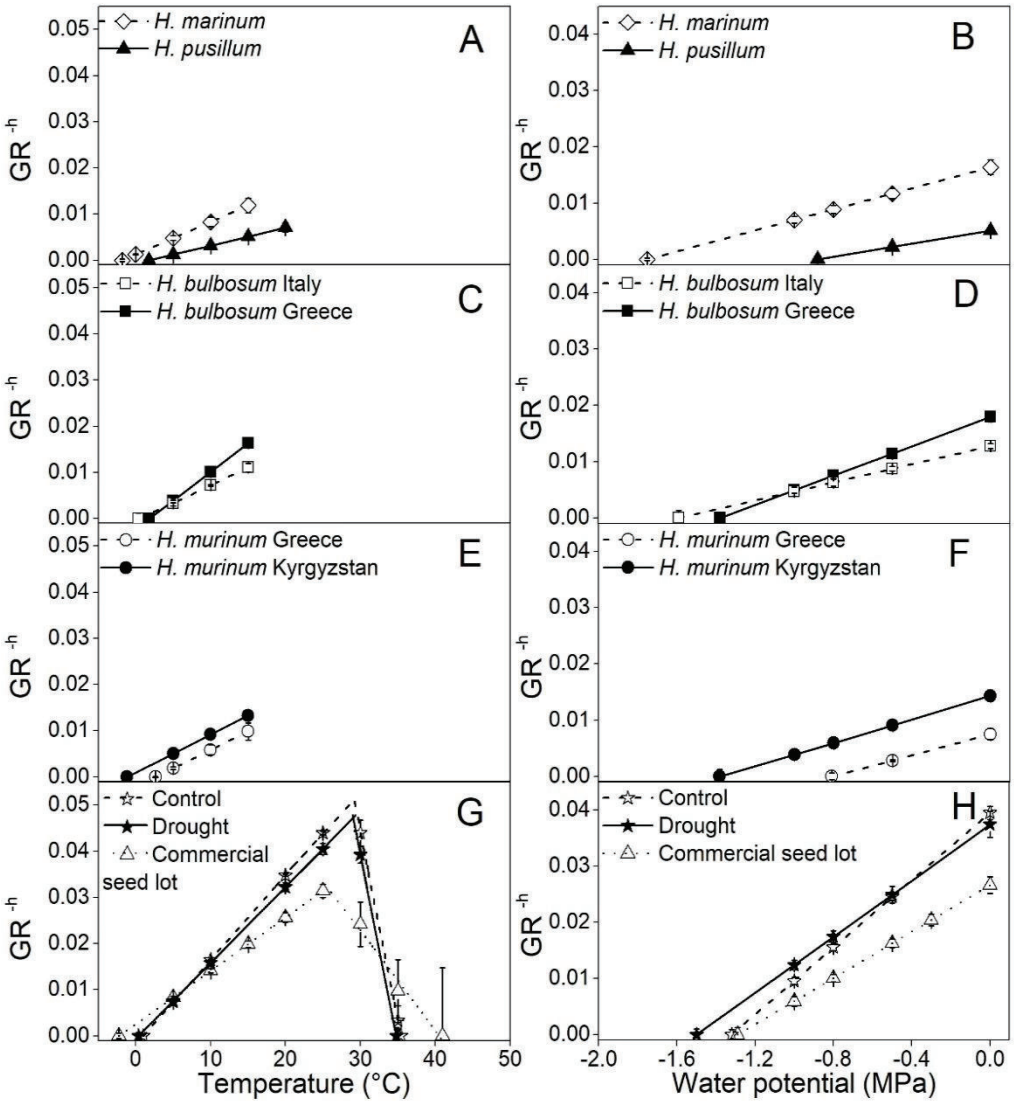


Figure 4.1: Thermal and hydro time model description for the *Hordeum* genus. The germination rate (GR) was plotted against temperature (A, C, E and G) to estimate the base temperature (T_b) (A, C and E), optimal (T_o) and ceiling (T_c) temperature (G). The GR was also plotted against water potential to estimate the base water potential (Ψ_b) (B, D, F and H) for six *Hordeum* CWRs, two water treatments (control and drought) and one commercial seed lot of the crop *H. vulgare*. The regression lines were calculated from the repeated probit analysis estimations and the error bars are the standard deviation (SD) of three replicates in CWRs and four replicates in crops, for each condition. For most of the data, the error bars were smaller than the symbols (A, B, C, D, E and F).

Table 4.1: Seed germination parameters of the *Hordeum* genus, six *Hordeum* CWRs and three crops seed lots, control and drought treatment and the commercial seed lot of the crop *H. vulgare*. The data were calculated using the repeated probit analysis and are the mean of three replicates for CWRs and four for crops for base temperature (T_b), thermal time (θ_T), ceiling temperature (T_c), supra-optimal thermal time (θ_{Tsupra}), base water potential (Ψ_b) and hydro time (θ_H). The hydrothermal time (θ_{HT}) was calculated from the means of the other parameters using Equation 2.9 from Chapter 2. Different letters indicate significant differences between CWRs ($P < 0.05$) and separately between crop seed lots.

CWRs	**T_b (°C)**	**θ_T (°Ch)**	**T_c (°C)**	**θ_{Tsupra} (°Ch)**	**Ψ_b (MPa)**	**θ_H (MPah)**	**θ_{HT} (MPa°Ch)**
H. marinum, Greece	-1.8 a	1448.7 a	-	-	-1.75 a	107.7 a	1989
H. bulbosum, Italy	0.3 ab	1347.7 a	-	-	-1.59 ab	124.7 a	2325
H. pusillum, Texas	1.8 b	2634.7 b	25.0	600.0	-0.88 c	174.3 b	2779
H. bulbosum, Greece	1.8 b	806.8 a	-	-	-1.38 b	77.0 a	1921
H. murinum, Greece	2.6 b	1306.0 a	33.5	3997.0	-0.81 c	110.3 a	1955
H. murinum, Kyrgyzstan	-1.2 a	1223.3 a	-	-	-1.38 b	97.3 a	1849
Crop *H. vulgare*							
Control treatment	1.0 c	546.0 c	35.4 a	123.8 a	-1.32 d	33.5 c	718
Drought treatment	0.4 c	608.9 d	34.8 a	123.0 a	-1.50 e	40.3 d	938
Commercial seed lot	-2.3 d	869.7 e	44.0 b	525.0 b	-1.29 d	48.8 e	1342

Normal seedlings: The seeds of CWRs were germinated under sub-optimal temperatures (5 to 20 °C) and water potentials (0 until -1.0 MPa) and all germinated seeds produced normal seedlings.

On the other hand, the seeds of the crops were germinated at sub- and supra- optimal temperatures (5 to 35 °C) and water potentials (0 to -1.0 MPa) and all the germinated seeds produced normal seedlings except at 35 °C and -1.0 MPa (Table 4.2). In these two conditions, percentage normal seedlings were significantly lower than percentage germination for all seed lots of *H. vulgare*.

Table 4.2: Conversion of germinated seeds into normal seedlings on *H. vulgare* seed lots. Germination (G %) and normal seedling percentages (S %) from seeds of the two crop treatments (control and drought) and the commercial seed lot of *H. vulgare* at six temperatures (T) and four water potentials (Ψ).

	Control		**Drought**		**Commercial seed lot**	
T (°C)	**G %**	**S %**	**G %**	**S %**	**G %**	**S %**
5	100	100	100	100	95	95
10	97	97	99	99	84	84
20	99	99	100	100	93	93
25	100	100	98	98	88	88
30	95	95	97	97	65	65
35	43	0*	16	0*	49	0*
Ψ (MPa)						
0	99	99	100	100	93	93
-0.5	100	100	99	98	88	88
-0.8	99	98	99	99	68	65
-1.0	88	43*	100	88*	65	53*

* normal seedlings did not develop or were significantly lower ($P < 0.05$) compared to seed germination.

4.2.2 Correlations between seed traits

The seed germination traits of CWRs and *H. vulgare* seed lots were compiled and correlations sought between them. Ψ_b was positively correlated with T_b ($r = 0.51$; $P < 0.01$ Figure 4.2 A), so that seed lots with lower T_b had lower Ψ_b values. Similarly, θ_T and θ_H had a positive correlation ($r = 0.87$; $P < 0.001$, Figure 4.2 B). Furthermore, θ_T and θ_H were correlated with θ_{HT} ($r = 0.83$ and $r = 0.93$ respectively, $P < 0.001$ in

both cases, Figure 4.2 C) as expected from the nature of the models used. However, θ_T and T_b or Ψ_b and θ_H were not correlated (*Appendix* Table A4.1).

With regard to the seed characterisation described in Chapter 3, θ_T and θ_H were negatively correlated with embryo length ($r = -0.81$ and $r = -0.91$ respectively, $P < 0.0001$, Figure 4.3A), endosperm length ($r = -0.85$ and $r = -0.84$ respectively, $P < 0.0001$, Figure 4.3 B) and mean seed mass ($r = -0.51$, $P < 0.01$ and $r = -0.70$, $P < 0.001$ respectively, Figure 4.3C). Thus, larger embryos and heavier seeds germinated faster (i.e. shorter θ_T and θ_H) than smaller embryos and lighter seeds.

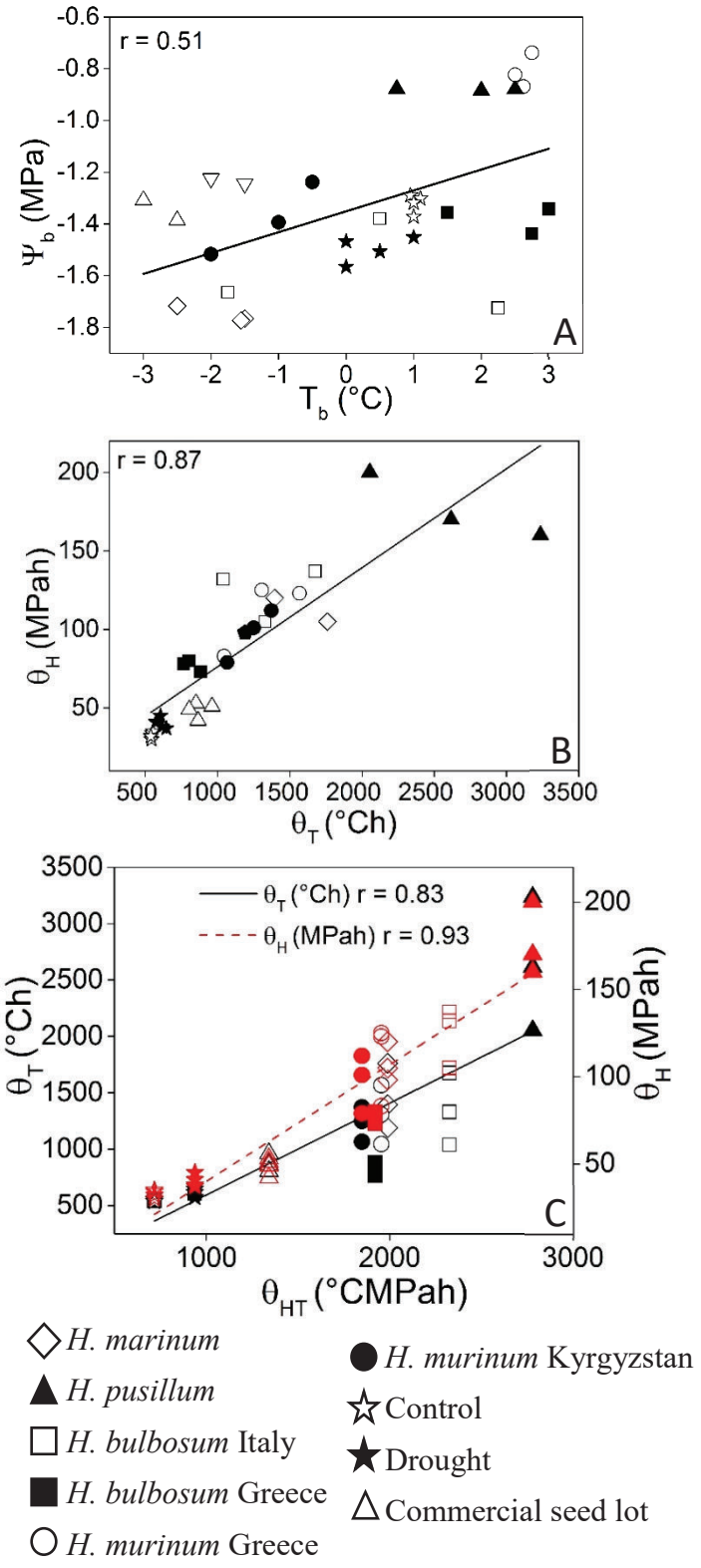


Figure 4.2: Correlations between seed germination traits of nine *Hordeum* populations (six CWRs and three crops). A) base water potential (Ψ_b) and base temperature (T_b) $P < 0.01$; B) hydro time (θ_H) and thermal time (θ_T) $P < 0.001$; and C) θ_T (black and solid line) and θ_H (red and dashed line) correlated with hydrothermal time (θ_{HT}) $P < 0.001$. Each point represents a replicate of each population, with three replicates for CWRs and four replicates for crops. DF = 25 for each correlation

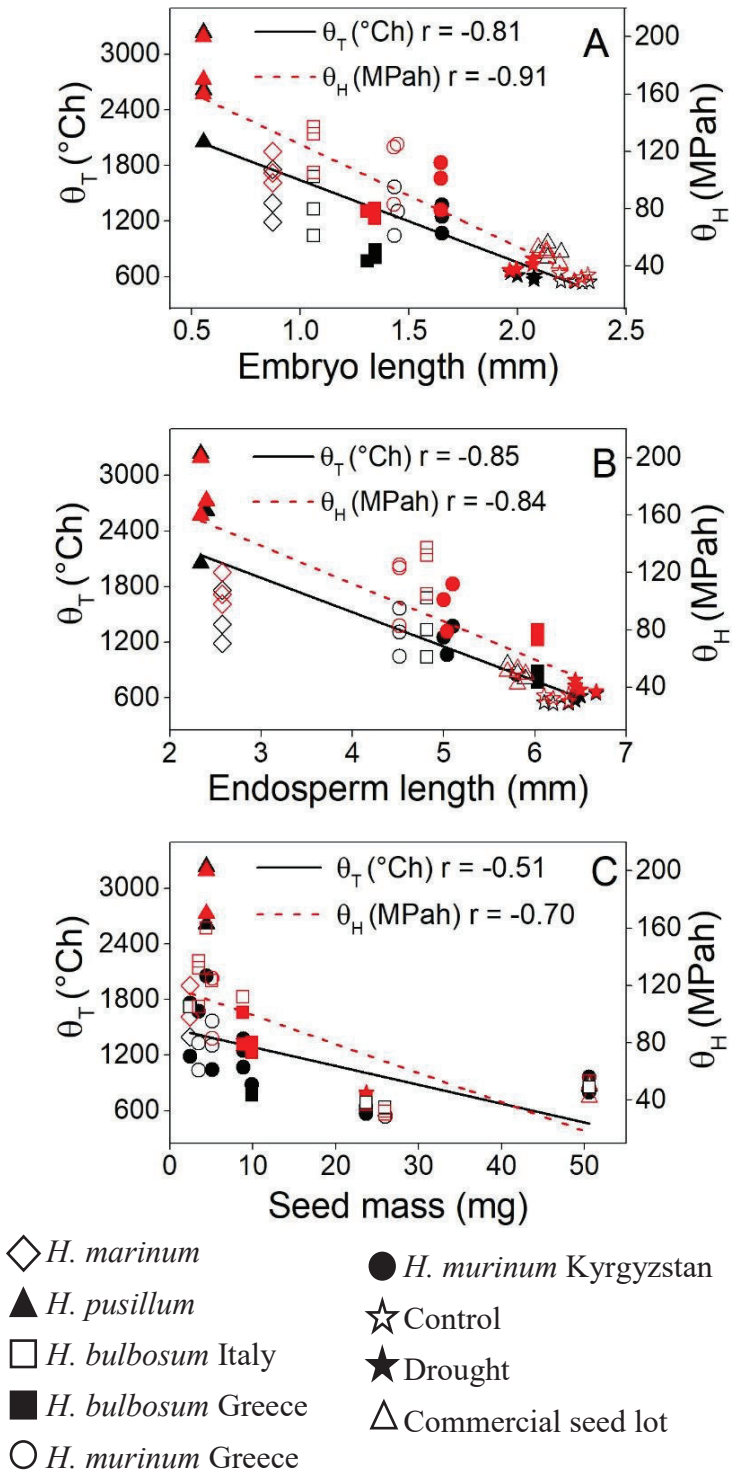


Figure 4.3 Correlations between seed germination traits and physical traits of nine *Hordeum* seed lots (six CWRs and three crops). Thermal time (θ_T, black and solid line) and hydro time (θ_H, red and dash line) were correlated with A) embryo length $P < 0.0001$; B) endosperm length, $P < 0.0001$ and C) mean seed mass, $P < 0.001$. Each point represents a replicate of each population, with three replicates for CWRs and four replicates for crops. DF = 25 for each correlation

4.2.3 Correlations between the environment of seed collection site and CWR seed traits

Correlations between the environment of seed collection site described in Chapter 2, Table 2.3 and seed functional traits of CWRs were carried out on the six *Hordeum* CWRs. Only one significant correlation was found. Higher mean maximum temperatures (annual and during the month of germination) were positively correlated with θ_T ($r = 0.53$; $P < 0.05$ and $r = 0.65$; $P < 0.01$ respectively, Figure 4.4). Thus, when the mean maximum temperature of the month of germination was higher, the θ_T values were larger indicating that seeds required a longer time to germinate on thermal basis. However, this correlation was only significant when the data of *H. pusillum* (with the longest θ_T) was included. Finally, the altitude was not correlated ($P > 0.05$, see *Appendix* Table A4.2) with any germination parameter; and seed functional traits did not correlate with the temperatures at other times of the year when plant developmental events such as flowering would have occurred.

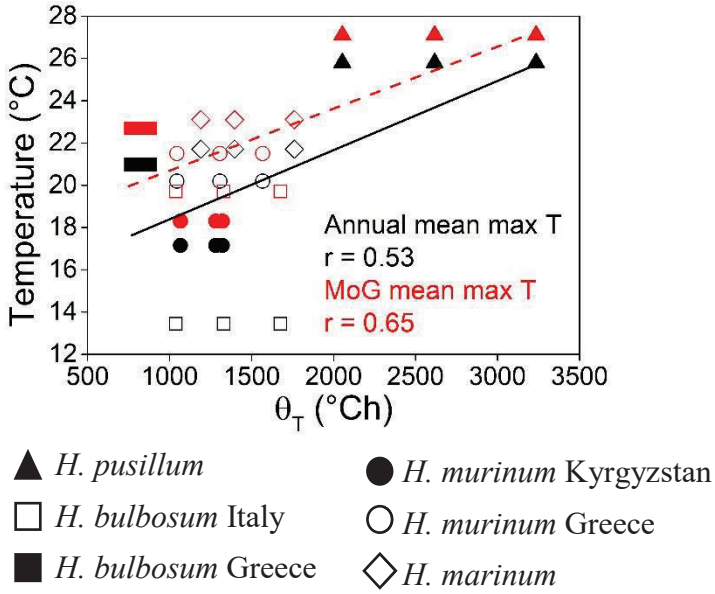


Figure 4.4: Correlations between the annual mean maximum temperature and thermal time (θ_T) of *Hordeum* CWRs. Annual mean maximum temperature (black solid line) and the mean maximum temperature during the month of germination (MoG) (red dash line) ($P < 0.05$ and $P < 0.01$ respectively). Each point represents a replicate of each population. DF = 16 for each correlation

4.3 Discussion

The diversity of germination performance in terms of thermal and hydro parameters for *Hordeum* seed lots (CWRs and crops) has been described in this chapter. This study has shown that the crop *H. vulgare* germinated faster than CWRs. Selection and breeding has led to larger seeds in many crops [e.g., bean, Maass (2006), maize and peas, Weeden (2007)]. Uniformity and homogeneity for seed germination is also an objective of breeding (Maass, 2006; Gepts, 2010) and is observed in this chapter where the crop seed lots germinated faster than the CWRs.

Seeds of *H. vulgare* produced by two different production water treatments showed differences in their seed germination traits. Seeds from the drought treatment needed more time to germinate than those of the control treatment (higher values of θ_T, θ_H and θ_{HT} Table 4.1), and therefore the former could be considered as possessing lower seed vigour. However, the International Seed Testing Association defined seed vigour as '*the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments*' (ISTA, 2017).

Since seeds of the drought treatment had the lowest Ψ_b, it cannot be described as low vigour due to a better response under low water potential. This tolerance to low water potential may have been acquired on the mother plant during the seed filling phase (Fenner, 2000; Farahani *et al.*, 2010).

All the germinated CWRs seeds developed into normal seedlings under different conditions of temperature and water potential. However, in crops, two exceptions were found, at 35 °C and -1.0 MPa conditions, percentage seed germination was significantly higher than the percentage of normal seedlings. Previous studies have identified lethal temperatures during germination and seedling emergence (from 30 to 40 ºC) of cereals such as, maize, wheat and rice (Yoshida, 1981; Porter & Gawith, 1999; Sánchez *et al.*, 2014). Yoshida (1981) found at 40 ºC or higher temperatures there was no cell division in the radicle tip. As cell division commences in the post-germination phase in cereals, these high temperatures might have a deleterious effect on normal seedling development (Bewley, 1997). Moreover, low water potential may also restrain cell division (de Castro *et al.*, 2000), and hence prevent normal seedling development. Seed germination percentage overestimated the number of normal seedlings of crops in extreme conditions (high temperatures and low water potentials). These results are consistent with Popova *et al.* (2013) and Ballesteros & Pence (2017) who also found discrepancies between seed germination and the number of normal seedlings of the Salicaceae family. Thus, it is necessary to emphasise the importance of recording seedling normality at the end of germination experiments when using extreme conditions such as supra-optimal temperatures or low water potentials. Seeds of the CWRs were more tolerant to low water potential than those of the crops since their germinated seeds were able to develop into normal seedlings.

Seed traits of CWRs and crops of the *Hordeum* genus were compiled and correlations sought among them. The positive correlation between T_b and Ψ_b suggest that species requiring higher temperatures to germinate also tended to require higher water potentials. Gardarin *et al.* (2010) found the same correlation between T_b and Ψ_b in 27 European weed species (from which seven species were from the Poaceae family). It is expected

that seed lots with low T_b and low Ψ_b have higher values of θ_T and θ_H. This is consistent with the positive correlation between θ_T and θ_H described here for the *Hordeum* genus. Plotting the values of θ_T and θ_H reported by Larsen *et al.* (2004) for three grass species showed a linear relationship between them. This suggests such a correlation is likely in more species.

Previous studies have found a positive relationship between seed size or seed mass and the speed of germination or seedling establishment (Jurado & Westoby, 1992; Leishman & Westoby, 1994; Westoby *et al.*, 1996; Leishman *et al.*, 2000; Moles & Westoby, 2004b). There is a specific case on the embryo length of *Hordeum* species, where seed with longer embryos germinated faster than those with shorter embryos (López-Castañeda *et al.*, 1996). Results from these previous reports agree with the negative correlation found here between θ_T and θ_H of *Hordeum* crops and CWRs and the mean seed mass, embryo length and endosperm length (Figure 4.3). In *Hordeum* seeds, the endosperm is a storage tissue that supplies the reserves necessary for subsequent seedling growth. As there appears to be no direct reliance, it is not obvious why larger endosperms are related to faster germination. Furthermore, the endosperm length in *Hordeum* seeds was not reflected in seed mass since there was no correlation between them (*Appendix*, Table A4.1).

Temperature and precipitation control the timing of germination in the wild. The mean maximum temperature of the month of germination and θ_T was positively correlated for the CWRs, showing that the seeds likely needed more thermal time to germinate at warmer temperatures. However, slow germination at warmer temperatures (month of germination) could be a strategy of the seeds to avoid exposing the seedlings to intense heat and high evapotranspiration due to warm temperatures. Moreover, the seeds that needed more time to germinate on a thermal time basis were produced at higher temperatures too (historical annual mean maximum temperature (Figure 4.4). Despite the seeds of *Hordeum* CWRs studied here did not show dormancy, plants of *Hordeum spontaneum* growing under high temperatures tend to produce seeds with deeper seed dormancy (Yan *et al.*, 2008) that might lead to slower seed germination. *H. pusillum*, had the longest θ_T and θ_H compared to the other *Hordeum* CWRs and it

was the only species from the American taxa (Petersen & Seberg, 2003). The other *Hordeum* CWRs were from the Eurasia taxa (*H. marinum, H. murinum* and *H. bulbosum*) and did not differ significantly in their θ_T and θ_H. However, these taxonomic distances were not reflected in T_b nor Ψ_b. This finding suggests that θ_T and θ_H could be more influenced by the genetic background than influenced by the environment of the seed collection site of *Hordeum* CWRs. More species from the American taxa would be needed to corroborate the influence of genetic background on the θ_T and θ_H for *Hordeum* CWRs.

5 CHAPTER 5: QUANTIFYING GERMINATION PERFORMANCE IN CROP WILD RELATIVES OF THE *BRASSICA* GENUS AND THE CROP *B. OLERACEA*

5.1 Introduction

Brassica is the most relevant genus of the Brassicaceae family because of its economic importance in agriculture (Tsunoda *et al.*, 1980). The *Brassica* CWRs have desirable traits such as drought and salt tolerance (Tsunoda *et al.*, 1980; Fahey *et al.*, 2001; Ozturk *et al.*, 2008; Kumar *et al.*, 2012). The genus is mainly distributed in Europe and the Mediterranean region. The CWRs of *Brassica* studied in this chapter were selected from different locations having different environments (precipitation and temperature).

In consideration of future agriculture in France, 36 cover crops (across six plant families including the *Brassicaceae*) have been characterised for germination functional traits (Tribouillois *et al.*, 2016). Four *Brassicas* (*B. juncea, B. rapa, B. napus, B. carinata*) had T_b of *c.* 7°C and three species had similar base water potentials, Ψ_b, for germination (-0.9 to -1 MPa), the exception being *B. rapa* (-2.2 MPa) (Tribouillois *et al.*, 2016). These findings lend weight to the argument that a species geographical origin defines the environmental conditions in which they can germinate (Cochrane *et al.*, 2014b; Dürr *et al.*, 2015).

The convergence of functional traits in species occupying similar environments can be considered an adaptation through environmental filtering (Keddy, 1992). Precipitation and temperature are the main environmental factors that have an impact on plant traits, e.g., leaf mass, yield, height and flowering time and seed traits, such as seed mass, germination and dormancy, show similar ecological associations (Dornbos & Mullen, 1991; Baskin & Baskin, 1998; Ackerly *et al.*, 2000; Peñuelas *et al.*, 2004; Porter, 2005; Menzel *et al.*, 2006; Franks *et al.*, 2007). Seed germination (i.e., radicle emergence) and seedling establishment are thought to be the most sensitive stage of a plant to environmental changes (Lloret *et al.*, 2004; Fay & Schultz, 2009; Kimball *et al.*, 2010).

Consequently, the effects of both precipitation and temperature on germination rate (Meyer *et al.*, 1990; Clauss & Venable, 2000; Levine *et al.*, 2008; Céspedes *et al.*, 2012), final germination (Alexander & Wulff, 1985; Gutterman, 2000b; Gareca *et al.*, 2012) and seedling establishment (Smith *et al.*, 2000; Lloret *et al.*, 2004; Jump *et al.*, 2008; Cochrane *et al.*, 2015a) have been widely explored.

Crop domestication has resulted in larger seed size and greater seed mass (Preece *et al.*, 2017) and they are also known to be responsive to environmental fluctuations (Roach & Wulff, 1987; Donohue *et al.*, 2005b; Nicotra *et al.*, 2010). In particular, seed filling is influenced by environmental conditions and mean seed mass of a population has been positively correlated with annual rainfall (Harel *et al.*, 2011) or with mean annual temperature (Murray *et al.*, 2004). Whilst seed size is thought to affect germination rate (Norden *et al.*, 2009), seed mass is rarely co-analysed with seed physiological traits of germination base temperature (T_b) or base water potential (Ψ_b). In this context, seed mass and T_b of tree seeds of *Aesculus hippocastanum* from across Europe had no correlation (Daws *et al.*, 2004), and a negative correlation was found between seed mass and Ψ_b of neotropical forest species (Daws *et al.*, 2008). In this chapter it was hypothesised that *Brassica* seeds from wetter environments (more precipitation) have higher seed mass and slower germination than those from drier environments.

The aim of this chapter is to investigate how the environment of seed collection site of *Brassica* CWRs (Chapter 2, Table 2.3) impacts on their germination functional traits. The assumption that current *Brasssica* crops may have been selected for fast germination under managed, irrigated conditions is also considered. Seed germination and the conversion into normal seedlings were analysed for the *Brassica* seed lots (Chapter 2, Table 2.3 and 2.4) at a range of temperatures and water potentials (Chapter 2, Table 2.6). The hypothesis is that warm temperatures and low water potentials can have detrimental effects on normal seedlings due to stopped cell division (Bewley, 1997). Seven geo-referenced CWRs (from across Europe, North Africa and the Middle East) were used. *Brassica* CWRs were selected from locations with different mean monthly precipitation (1

– 94 mm) and annual mean temperatures (minimum of -1.6 and maximum of 34 °C). Seeds from two experimental treatments (produced under non-limiting and water limited conditions) of two crop research genotypes (seeds with low and high vigour) of *B. oleracea* (see Chapter 2 section 2.2.3) and one commerical seed lot were also studied. From the crop *B. oleracea* seed lots it was hypothesised that 1) the seeds of the high vigour genotype germinate faster i.e. shorter θ_T and θ_H, than those of low vigour genotype, and 2) the seeds of the drought treatment have lower Ψ_b than those of the control treatment.

The environmental information (temperature and precipitation) of the *Brassica* CWRs was obtained from WorldClim (Chapter 2, section 2.2.2)

The month of germination was considered to be the first month after the seed dispersal month that the following assumptions were met: (1) the minimum rainfall required for germination (mean monthly of 15 mm; Freas and Kemp, 1983; Gutterman, 1993; Gutterman, 2000a) and (2) the temperature exceeded T_b but did not exceed T_c (*Appendix* Figure A5.1). The exception was *B. rapa* subsp. *sylvestris* from Algeria where the maximum monthly precipitation did not exceed 2 mm (*Appendix* Figure A5.1). For this species, the first period of rainfall was taken as the precipitation threshold. Furthermore, relationships between seed gemination traits and seed morphology, seed mass and oil content (described in Chapter 3) are explored. Finally, conclusions were drawn on the impact of the environment of collection site on subsequent seed germination traits of CWRs studied in the laboratory.

5.2 Results

5.2.1 Germination trait parameters

Seed germination of the 12 *Brassica* seed lots (seven CWRs and five crop seed lots of *B. oleracea*) showed a *c.* 9 °C range in each of the cardinal temperatures: T_b from 1.7 to 10.5 °C; T_o from 25 to 35 °C; and T_c from 36 to 45 °C (Figures 5.1A, C, E and G, Table 5.1). Intra-specific variability in trait parameters was observed in two wild seed lots of *B. rapa* from France and Switzerland (Figure 5.1C) and two wild seed lots of *B. rapa*

subsp. *sylvestris* from Morocco and Algeria (Figure 5.1E), with significant differences ($P < 0.05$) in germination rate (GR) and T_b (Table 5.1). Thermal time (θ_T) values also differed three-fold amongst CWRs (from 214.7 °Ch for *B. rapa* subsp. *sylvestris* from Morocco to 775.5 °Ch for *B. rapa* from Switzerland). The crop research genotype *B. oleracea* A12DHd had the longest θ_T (control 951.6 °Ch and drought 953.4 °Ch Table 5.1).

In general, seeds of the crop research genotypes A12DHd and AGSL101 were slower to germinate than the CWRs in terms of θ_T in the sub-optimal range of temperatures. There were no significant differences between the water treatments in either of the crop research genotypes (Table 5.1). The high vigour crop genotype, AGSL101, had faster germination (i.e. shorter θ_T, $P > 0.01$) than the low vigour crop genotype, A12DHd; whilst the commercial seed lot had θ_T very similar to the CWR *B. rapa* subsp. *campestris* (i.e., *c.* 445 °Ch). In contrast, T_b for the *B. oleracea* seed lots did not differ (Figure 5.1G; Table 5.1). However, T_c was higher in the crop genotype AGSL101 and the commercial lot than the low vigour crop genotype A12DHd (40 °C and 41.5 °C vs 36 °C, respectively, $P < 0.001$). In the supra-optimal temperatures, θ_{Tsupra} was shorter in the commercial seed lot, but the water treatments of the two research genotypes did not differ.

Seeds were also germinated under five water potentials. GR was lower and final germination decreased with more negative water potentials, and Ψ_b values varied between -0.4 MPa and -2 MPa (Figure 5.1B, D, F and H). The seed lots of the CWR *B. rapa* had the lowest Ψ_b of -1.59 MPa and -1.94 MPa (France, Switzerland respectively; Figure 5.1D). The hydro time (θ_H) values also differed among CWRs from 9.0 MPah for *B. rapa* subsp. *campestris* to 72 MPah of *B. rapa* Switzerland (Table 5.1). At the end of the experiment, all non-germinated seeds were transferred to water at the same temperature defined in Chapter 2, Table 2.6, and subsequently germinated within 48 hours, except for *B. rapa* subsp. *sylvestris* (Algeria) which only achieved 40 % germination after 15 days. However, a tetrazolium test indicated that all the seeds of *B. rapa* subsp. *sylvestris* (Algeria) were viable.

Whilst the θ_H of the crop research genotypes and the commercial lot was within the range of the CWRs (Table 5.1), Ψ_b of the crop seed lots was higher (less negative) than the CWRs, except for *B. tournefortii* and *B. rapa* subsp. *campestris*. Within the crop seed lots, the commercial lot of *B. oleracea*, had the smallest θ_H (14.3 MPah). There were no significant differences between low and high vigour genotypes nor between water treatments (control and drought, Table 5.1). In contrast, the Ψ_b was significantly lower ($P < 0.05$) in both AGSL101 (control and drought) and the commercial seed lot than in A12DHd (Table 5.1). Therefore, seeds of the high vigour genotype, AGSL101, and the commercial seed lot could germinate over a wider range of water potentials than the low vigour genotype (Figure 1H).

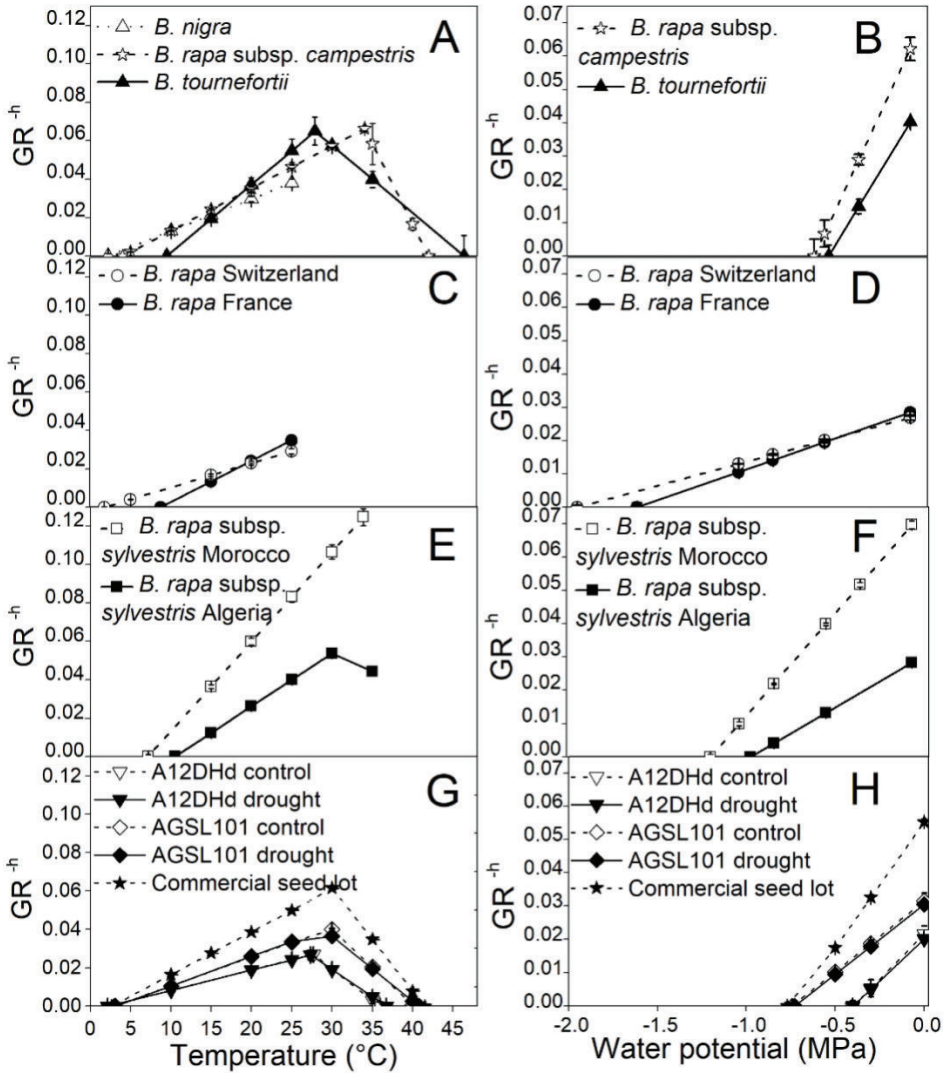


Figure 5.1 Thermal and hydro time model description for the *Brassica* genus. The germination rate (GR) was plotted against temperature (A, C, E and G) to estimate the cardinal temperatures and the water potential thresholds (B, D, F and H) of seven *Brassica* CWRs, two research genotypes of *B. oleracea*, subjected to water treatments (control and drought) and one commercial seed lot. The seeds were germinated under a range of sub and supra optimal temperatures between 5 to 40 °C and on PEG solutions at 0, -0.3, -0.5, -0.8 and -1.0 MPa at one constant temperature (20 or 25 °C, depending on the species). For most of the data, the error bars were smaller than the symbols and therefore not shown (C, D, E, F, G and H).

Table 5.1 Seed germination parameters of the *Brassica* genus. Seven *Brassica* CWRs and two research crop genotypes (A12DHd low vigour and AGSL101 high vigour) subjected to controlled water treatments (control and drought) and one commercial seed lot of the crop *Brassica oleracea*. The data was calculated using the repeated probit analysis and are the mean of three replicates for CWRs and four for crops for base temperature (T_b), thermal time in the sub-optimal range of temperatures (θ_T) and supra-optimal range of temperatures (θ_{Tsupra}), ceiling temperature (T_c), base water potential (Ψ_b) and hydro time (θ_H). The hydrothermal time (θ_{HT}) was calculated using the mean of the other parameters using Equation 2.9 from *Chapter 2*. Different letters mean significant differences between CWRs and separately, between crop seed lots ($P < 0.05$).

CWRs	**T_b (°C)**	**θ_T (°Ch)**	**T_c (°C)**	**θ_{Tsupra} (°Ch)**	**Ψ_b (MPa)**	**θ_H (MPah)**	**θ_{HT} (MPa°Ch)**
B. rapa, Switzerland	1.8 c	775.5 a	-	-	-1.94 c	72.0 a	1567
B. nigra, England	2.2 c	593.6 b	-	-	-	-	-
B. rapa, France	7.5 b	524.9 bc	-	-	-1.59 d	56.0 b	1281
B. rapa subsp. *campestris*, Turkey	4.0 c	449.4 c	42.00 b	120.4 b	-0.56 a	9.0 d	318
B. rapa subsp. *sylvestris*, Morocco	7.2 b	214.7 d	47.50 a	109.2 b	-1.17 b	16.7 d	452
B. tournefortii, Egypt	10.2 a	254.3 d	45.33 ab	268.5 a	-0.47 a	11.8 d	238
B. rapa subsp. *sylvestris*, Algeria	10.5 a	363.1 c	-	-	-0.93 b	33.0 c	761
Crop *B. oleracea*							
A12DHd control	2.1 d	951.6 e	36.00 c	317.5 cd	-0.40 e	18.8 ef	1101
A12DHd drought	2.1 d	953.4 e	36.74 c	356.3 c	-0.40 e	20.0 ef	1271
AGSL101 control	3.0 d	665.2 f	40.20 d	255.0 de	-0.74 f	23.8 e	765
AGSL101 drought	3.1 d	648.0 f	40.79 d	296.3 d	-0.73 f	24.0 e	798
Commercial seed lot	2.8 d	442.4 g	41.50 d	187.4 e	-0.78 f	14.3 f	357

Normal seedlings.

All the seeds of CWRs that germinated under sub-optimal temperatures (5 to 25 °C) and water potentials (0 to -1.0 MPa) produced normal seedlings. The seed germination at supra-optimal range of temperatures was only performed in four CWRs, *B. rapa* subsp. *campestris*, two seed lots of *B. rapa* subsp. *sylvestris* and *B. tournefortii* due to limited number of seeds. There were significant differences between seed germination and normal seedlings in the CWRs seed lots germinated in the supra-optimal range of temperatures (Table 5.2). All four seed lots did not develop normal seedlings above 35 ºC even though two of them germinated at 40 ºC (Table 5.2). *B. tournefortii* was the only CWRs that have significant reduction of normal seedlings at 25 ºC.

Table 5.2 Conversion of germinated seeds into normal seedlings of four *Brassica* CWRs. Percentage seed germination (G %) and normal seedlings (S %) of four *Brassica* CWRs subjected to a wide range of temperatures (T) imbibed in water. The values are the mean of three replicates.

	B. rapa subsp. *campestris*		*B. rapa* subsp. *sylvestris* (Morocco)		*B. rapa* subsp. *sylvestris* (Algeria)		*B. tournefortii*	
T (°C)	**G %**	**S %**	**G %**	**S %**	**G %**	**S %**	**G %**	**S %**
10	84	84	-	-	-	-	10	10
15	88	85.3	100	100	45.3	45.3	65	60
20	93.3	93.3	100	98.7	89.3	85.3	96	96
25	98	89.3	100	97.3	96	96	97	40*
30	97	80	97.3	49.3*	100	98.7	95	4*
35	98	8*	100	4*	100	0*	65	0*
40	50	0*	98.7	0*	-	-	-	-
42	0	0	0	0	-	-	-	-

* normal seedlings did not develop or their percentages were significantly lower ($P < 0.05$) than percentage seed germination.
- temperatures non-tested

The seeds of the research crop genotypes germinated at supra-optimal temperatures had poor conversion into normal seedlings (Table 5.3). These differences also existed when germinated at low water potentials. Similar to *Brassica* CWRs described above, the research crop genotypes did not

produce normal seedlings at 35 ºC nor 40 ºC. However, contrary to CWRs, the crops had poor conversion between germinated seeds and normal seedlings at low water potentials (-1.0 MPa, Table 5.3). On the other hand, the high vigour genotype (AGSL101) did not differ in the conversion of germinated seeds into normal seedlings with the low vigour genotype (A12DHd) when the seeds were germinated under a temperature range. However, the seeds of the high vigour genotype (AGSL101) were able to germinate and convert germinated seeds into normal seedlings at lower water potentials (-0.8 MPa and -1.0 MPa) than the low vigour genotype. In general, germinated seeds of the commercial seed lot developed into normal seedlings under all the conditions (temperature and water potentials) with the exception of 40 ºC where there was no conversion into normal seedlings (data not shown).

Table 5.3 Conversion of germinated seeds into normal seedlings of *Brassica oleracea*. Percentage seed germination (G %) and normal seedlings (S %) of four crop seed lots, two research crop genotypes (A12DHd low vigour and AGSL101 high vigour) subjected to water treatments (control and drought) of *B. oleracea* at several temperatures (T) and water potentials (Ψ). The values are the mean of four replicates.

	A12DHd control		A12DHd drought		AGSL101 control		AGSL101 drought	
T (°C)	**G %**	**S %**	**G %**	**S %**	**G %**	**S %**	**G %**	**S %**
10	100	100	98.9	98.9	97.9	96.9	99	99
20	99	97	97	97	99	99	99	97
25	95	94	97	97	96.9	94.8	98	94
30	93.9	86.9	91.1	89.1	100	99	97	97
35	45	0*	84.8	0*	93.8	0*	92	0*
40	3	0	1	0	3	0	15	0*
Ψ (MPa)								
0	95	94	95	94	96.9	94.8	98	94
-0.5	91	86	93	91	100	98	95.8	92.6
-0.8	27	0*	33.3	0*	97	80.8	96	75
-1.0	7	0*	12	0*	39.4	23.2	42.4	19.2*

* normal seedlings did not develop or their percentages were significantly lower ($P < 0.05$) than percentage seed germination.

5.2.2 Correlations between seed traits

The seed traits of all *Brassica* seed lots (including both crop and CWRs) were compiled and subjected to linear regression models. A negative correlation was found between T_b and θ_T for all seed lots tested ($P < 0.0001$; $r = -0.72$, Figure 5.2A). Ψ_b and θ_H were also negatively correlated ($P < 0.0001$; $r = -0.88$ Figure 5.2B). The lower the base threshold (temperature or water potential) the longer the germination process (θ_T or θ_H). With regard to the seed characterisation described in Chapter 3, mean seed mass was correlated with θ_T and T_b (Figure 5.2C and D), indicating that heavier seeds need to accumulate more θ_T to germinate and had a lower T_b.

Regarding the seed morphology described in Chapter 3, the embryo length and the thickness of the seed coat were correlated with the T_b and θ_T (Figure 5.3A and B). *Brassica* seeds with longer embryos and thicker seed coats had lower T_b and longer θ_T than seeds with shorter embryos and thinner seed coats. Furthermore, the θ_H and Ψ_b were correlated with the oil content for the 12 seed lots (Figure 5.3C). Seeds with higher oil content had longer θ_H and lower (more negative) Ψ_b than seeds with lower oil content.

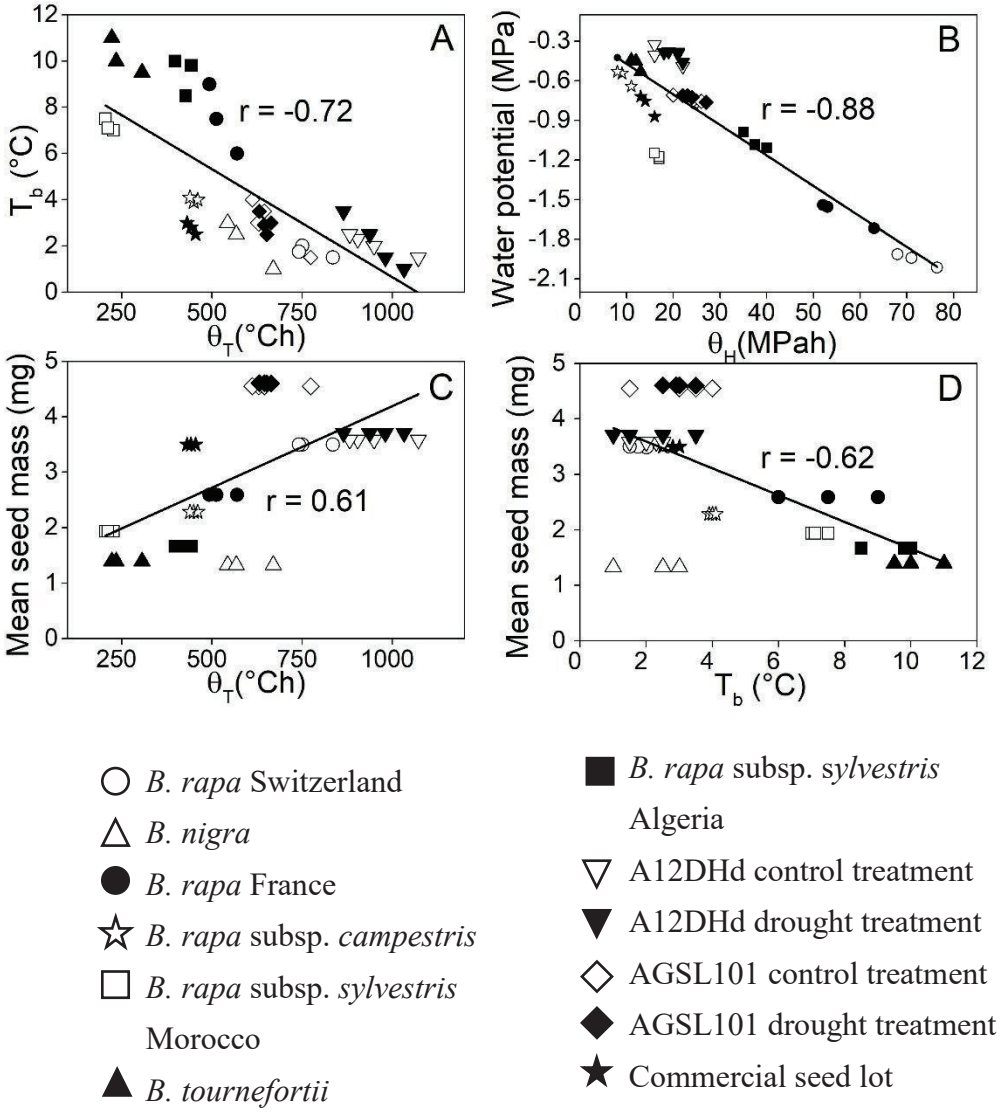
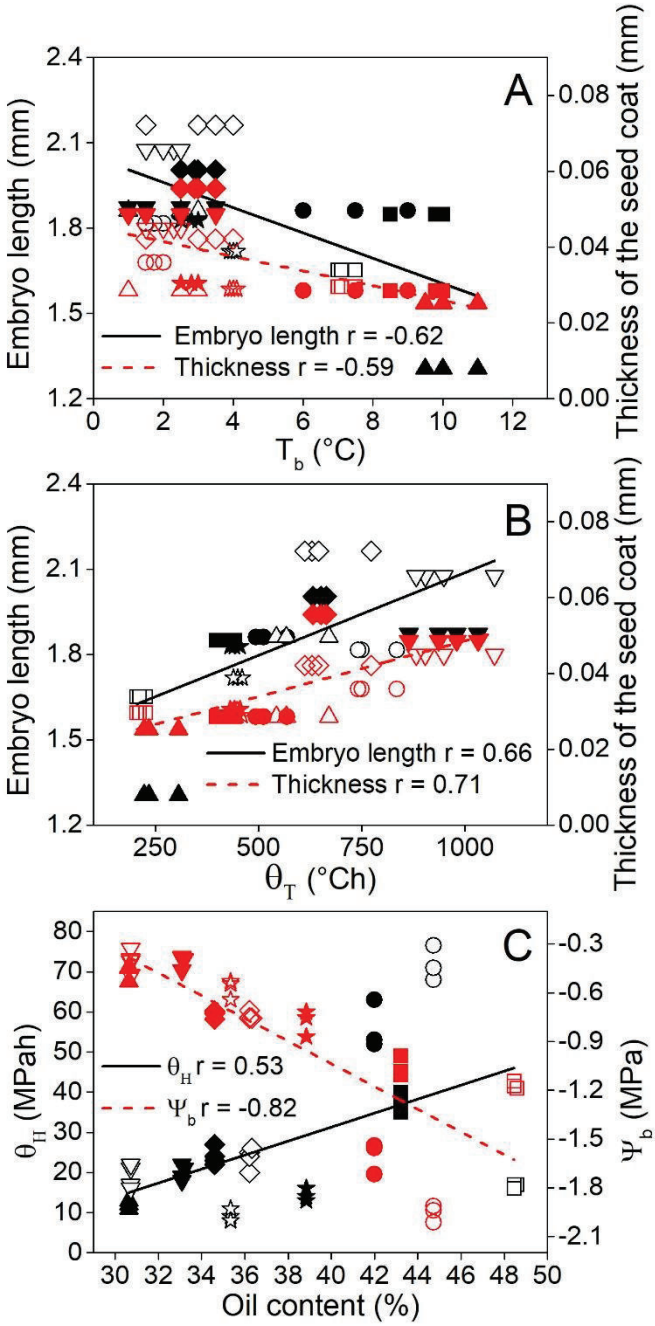


Figure 5.2 Correlations between seed traits of 12 *Brassica* seed lots (7 CWRs and 5 crops). A) Thermal time (θ_T) and the base temperature (T_b); B) hydro time (θ_H) and base water potential (Ψ_b); C) and D) correlations between the mean seed mass (weight of 100 individual seeds) with θ_T and T_b respectively. Each point represents a replicate of each seed lot, three replicates for CWRs and four replicates for crops. All the correlations had a significance of $P < 0.001$. DF = 34 in A, C and D and 31 in B



○ *B. rapa* Switzerland
△ *B. nigra*
● *B. rapa* France
☆ *B. rapa* subsp. *campestris*
□ *B. rapa* subsp. *sylvestris* Morocco
▲ *B. tournefortii*
■ *B. rapa* subsp. *sylvestris* Algeria
▽ A12DHd control treatment
▼ A12DHd drought treatment
◇ AGSL101 control treatment
◆ AGSL101 drought treatment
★ Commercial seed lot

Figure 5.3 Correlations between seed germination traits and physical seed traits of 12 *Brassica* seed lots (seven CWRs and five crops). Embryo length (black symbols and solid line) and thickness of the seed coat (red symbols and dash line) were significantly correlated ($P < 0.001$) with A) Base temperature (T_b) and B) thermal time (θ_T); C) hydro time (θ_H, black symbols and solid line) and base water potential (Ψ_b, red symbols and dash line) were correlated with oil content $P < 0.001$ in both cases. Each point represents a replicate of each population, with three replicates for CWRs and four replicates for crops. DF = 34 in A and B and 31 in C for each correlation.

5.2.3 Correlations between the environment of seed collection site and CWR seed traits

The relationship between CWRs seed functional traits and the environment of the seed collection site were assessed for the seven *Brassica* CWRs. T_b was lower when the mean monthly precipitation, and the precipitation of the predicted month of germination, were higher (Figure 5.4A). Seed lots of species from wetter environments had slower germination (i.e., longer θ_T; Figure 5.4B). Both precipitation measurements were correlated with Ψ_b and θ_H (Figure 5.4C and 5.4D). With regard to temperature, T_b and the annual mean temperature (minimum, mean and maximum) were correlated (*Appendix* Table A5.1). Finally, the altitude was not correlated ($P > 0.05$) with any germination parameter; and seed functional traits did not correlate with the temperatures at other times of the year when plant developmental events such as flowering would have occurred.

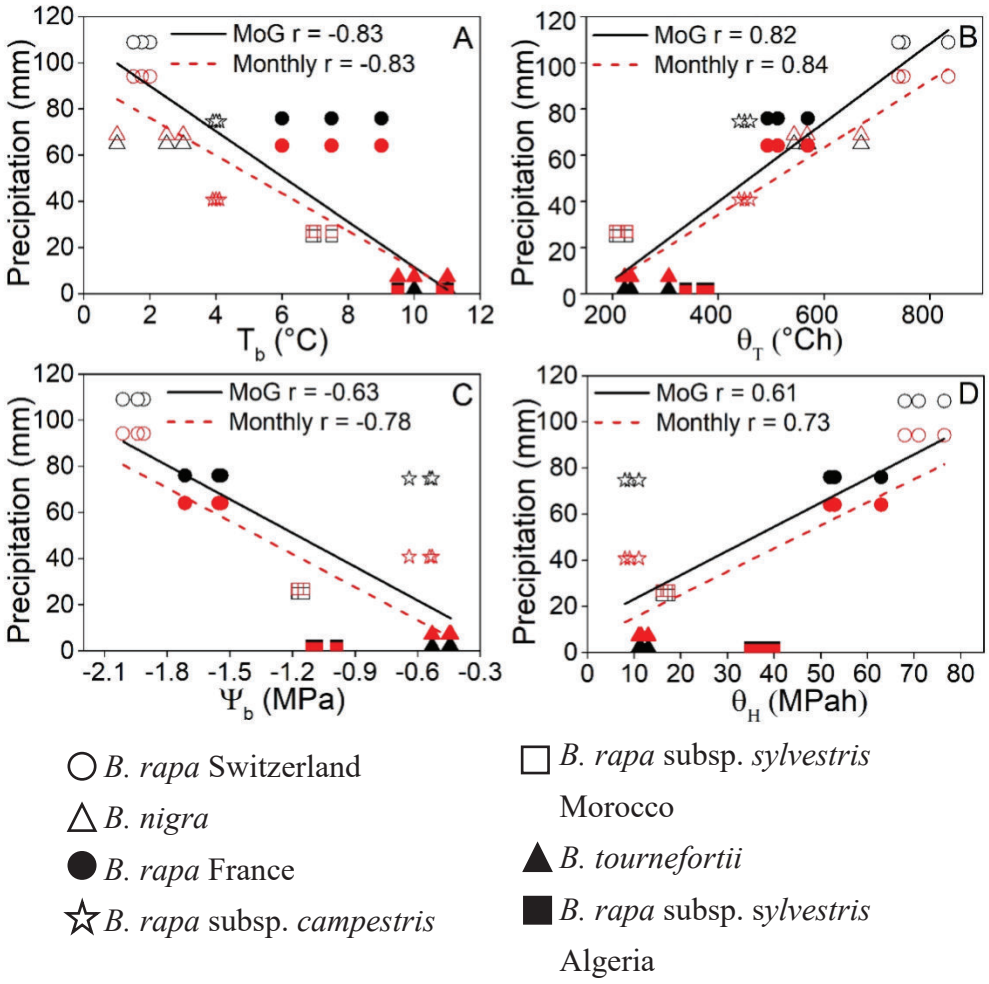
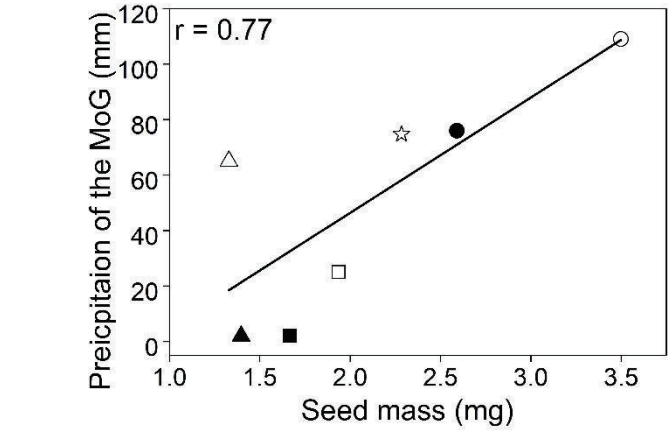


Figure 5.4 Correlation of the seed germination traits of seven *Brassica* CWRs and the environment of the seed collection site. Historical mean monthly precipitation (red symbols and dash line) and the precipitation of the month of germination (MoG, black symbols and solid line) were plotted against the base temperature (T_b A), the thermal time, (θ_T, B), the base water potential (Ψ_b, C) and the hydro time (θ_H, D). Each point is one of the three replicates of each seed lot. The correlations had a significance of $P < 0.001$ with the mean monthly precipitation and $P < 0.01$ with the MoG. DF = 19 in A and B and 16 in C and D for each correlation.

Only one correlation was found between the physical seed traits characterised in Chapter 3 and the month of germination environment of CWRs (Figure 5.5). The mean precipitation of the month of germination was positively correlated with the mean seed mass of *Brassica* CWRs. This correlation suggests that seeds from wetter environments are heavier than seeds from drier environments.



○ *B. rapa* Switzerland
△ *B. nigra*
● *B. rapa* France
☆ *B. rapa* subsp. *campestris*
□ *B. rapa* subsp. *sylvestris* Morocco
▲ *B. tournefortii*
■ *B. rapa* subsp. s*ylvestris* Algeria

Figure 5.5 Significant correlation between the mean precipitation of the month of germination, MoG, of the seed collection site environment and seed mass of seven *Brassica* CWRs ($P < 0.05$, $DF = 5$). Each symbol represents one seed lot.

5.3 Discussion

The diversity of germination performance in terms of thermal and hydro traits for *Brassica* seed lots (CWRs and crops) has been described in this chapter. This diversity is due to both, their genetic variability (Arias & Pires, 2012; Arias *et al.*, 2014) and the influence of the environment (phenotypic plasticity) (Schmid & Dolt, 1994; Mousseau & Fox, 1998; Van Kleunen *et al.*, 2007). In this study, I cannot definitively distinguish between genetic or phenotypic variability in the CWRs. However, by studying two *Brassica* seed lots, each from different environments, along with different species from across a broad environmental range, it was possible to assess the influence of the environment of seed collection site on seed functional traits. Moreover, studying two *B. oleracea* research crop genotypes selected for differences in vigour (A12DHd and AGSL101) grown under identical conditions, and same water treatments (control and drought), it enabled to consider the impact of limiting water during the seed filling stage and high vigour alleles in these research lines on germination traits.

The variation in *Brassica* germination thresholds (*c.* 9 °C) amongst 12 seed lots is wider than that observed for other comparative studies based on taxonomy or habitat. For example, in cold and warm season grasses, T_b ranges by 2.6 °C to 5.9 °C, respectively (Jordan & Haferkamp, 1989). Pasture species (e.g., clover, ryegrass and chicory) also display a relatively narrow T_b range of 3.5 °C (Moot *et al.*, 2000). In more than 50 species of Cactaceae, sampled across an environmental envelope that covers 70° of latitude, seed T_b varies by *c.* 20 °C (Seal *et al.*, 2017). The maternal environment seems to influence the expression of this trait, the wide variation in temperature thresholds in CWRs of *Brassica* could be explained by the selection of taxa from seven countries with widely differing environments. These environments have annual mean temperatures varying from 5 to 26 °C, across a latitudinal span of *c.* 20°.

The range of Ψ_b for the 50th germination percentile was also wide for species in the *Brassica* genus (i.e., -1.54 MPa) from -0.40 to -1.94 MPa.

This range looks wider because it is from only one genus when comparing with the range of several crops from different genera and families [such as, *Daucus carota* (Apiaceae), *Helianthus annuus* (Asteraceae) *Hordeum vulgare* and *Zea mays* (Poaceae)], that have a range extending to -2.1 MPa (Dürr *et al.*, 2015). Interestingly, *B. rapa* has been estimated to have a Ψ_b as low as -2.2 MPa (Tribouillois *et al.*, 2016). However, that study used data for only the 20th and 30th germination percentiles as viability was poor. In this chapter, average Ψ_bs for the same germination percentiles (20th) were -2.2 and -1.99 MPa for *B. rapa* from Switzerland and France, respectively which is similar to what they found.

Radicle emergence is the end of the germination phase but the conversion of germinated seeds into normal seedlings is another critical step to becoming an adult plant. ISTA (2017) uses normal seedlings as well as seed germination (i.e. radicle emergence) to assess seed vigour. Germinated seeds of *Brassica* seed lots developed into normal seedlings when germinated at sub-optimal temperatures in CWRs and crops. However, in the supra-optimal range of temperatures, seed germination overestimated normal seedlings, especially at 30 °C and 35 °C in CWRs and 35 °C in the research crop genotypes. Overestimation of normal

seedlings by reporting only radicle emergence has been discussed previously in other families such as Salicaceae and Asteraceae (Gay *et al.*, 1991; Popova *et al.*, 2013; Ballesteros & Pence, 2017) and in the *Hordeum* genus (Chapter 4 section 4.3). Moreover, seeds of the low vigour genotype (A12DHd) germinated at low water potentials did not produce normal seedlings. In this case the high vigour genotype (AGSL101) exhibits better performance in the conversion from seed germination into normal seedlings. Thus, seed germination percentage overestimated the number of normal seedlings of crops in extreme conditions tested. Therefore, normal seedlings have greater importance when subjected to high temperature or water stress in the seed bed.

Selecting for seed performance in Brassica

Seed quality is an essential trait for crop production and food security (Finch-Savage & Bassel, 2015). As a consequence, the seed industry strives to produce seed lots with enhanced performance, particularly vigour which is often only realised under non-optimal conditions. Vigour is a property of the seed that determines performance in a wide range of environments (ISTA, 2017). Amongst the 12 *Brassica* seed lots assessed over many temperature and water potential conditions I show strong correlations between both T_b and θ_T and between Ψ_b and θ_H (Figure 5.2), indicating the importance of determining these germination features to compare seed performance. These parameters were correlated with the maternal environment (of seed collection site) in the *Brassica* CWRs in addition to describe the seed population thresholds for temperature and water potential and the speed of germination on a thermal and water basis.

A relationship between Ψ_b and θ_H is expected, as the hydro time model shows that germination rate is inversely proportional to the difference between the actual water potential (Ψ) and the Ψ_b (Bradford, 1995). Thus, seeds with lower Ψ_b (more negative water potentials) will require more hydro time to germinate, i.e., have longer θ_H (Bradford, 1995). On the other hand, the correlation between T_b and θ_T has been reported previously for a range of different species (Trudgill *et al.*, 2000; Trudgill *et al.*, 2005; Gardarin *et al.*, 2011; Dürr *et al.*, 2015; Seal *et al.*, 2017). This might reflect ecological adaptation such that the seeds with a high threshold (T_b)

then proceed to germinate faster, i.e., shorter thermal times (Trudgill *et al.*, 2005; Gardarin *et al.*, 2011). As there is intra- and inter-specific variation in the thresholds for seed germination progress under a wide range of (thermal and water potential) environments (Dürr *et al.*, 2015), it is critical that these parameters are determined for each seed lot so that vigour can be more accurately described.

In general, the CWRs had lower Ψ_bs than the crop genotypes and hence had a wider window for low water potential tolerance. This suggest they may be able to take better advantage of rain when it occurs in smaller quantities and lower frequency. On the contrary, the selection of the crop *B. oleracea* for growth under optimal, irrigated monoculture agricultural conditions may have increased the risk of crop failure under future climates if water availability is more variable. The research crop genotypes had lower tolerance to drought conditions (i.e., the highest Ψ_b, Table 5.1) than the CWRs. This narrow range of water potentials over which germination can occur confirms their relative lack of vigour and response to abiotic stress compared with the CWRs.

The CWRs tended to have similar θ_T compared to the crops. Therefore, the selection and breeding of *B. oleracea* has not resulted in particularly faster germination in terms of thermal time, based on the seed lots characterised here. Even though crop genotype AGSL101 was the product of the introgression of two high vigour alleles, the impact of this on thermal time was not beneficial compared with CWRs, but it was beneficial compared with the low vigour genotype. A12DHd had the longest θ_T compared with the other crops and CWRs, i.e. germination is slower, which would increase the risk of inclement drought or seedbed deterioration impacting on the more slowly emerging seedlings. Such subtle differences on thermal and hydro-time characteristics amongst a range of *Brassica* seed lots with largely similar genetic backgrounds (Table 5.1, Dürr et al., 2015; Tribouillois et al., 2016) tends to suggest a continuum of responses within the genus.

Mean seed mass was positively correlated with θ_T, so that heavier seeds germinated more slowly (longer θ_T) than lighter seeds. This is in agreement with Grime *et al.* (1981) who studied 400 species and reported a decrease

of germination rate with increased seed weight. Norden *et al.* (2009) found a similar correlation between the mean seed mass and the mean time to germination (MTG) in more than 1000 tropical forest trees. The mean seed mass was also negatively correlated with T_b. Thus, small seeds should require less time to germinate because they need to accumulate less heat units above a higher T_b. Nonetheless, when both research genotypes were compared, seeds of heavier mass (high vigour genotype) germinate faster than seeds of lighter mass (low vigour genotype), but this is contrary to the general rule as demonstrated by the CWRs (Figure 5.2C). Crops are selected to have heavier seeds and faster germination, thus they may not conform to what is observed in the wild environment. Counterintuitively, seeds of CWRs germinated faster based on thermal time characteristics compared to crops, therefore it seems that there is a window to breed *Brassica* crops to obtain faster germination.

Brassica seeds with shorter embryos germinate faster on a thermal time basis than those with longer embryos. Furthermore, seeds with shorter embryos also had thinner seed coats (Chapter 3, Figure 3.4) which might allow the seeds to imbibe faster than seeds with longer embryos and thicker seed coats. Thus, the germination process could finish more quickly (Nooden *et al.*, 1985; Kikuzawa & Koyama, 1999; Gutterman, 2000a). This finding disagrees with *Hordeum* seeds (Chapter 4) and López-Castañeda (1996) where bigger seeds (higher seed mass and longer embryos) tend to germinate faster. In addition, *Brassica* seeds with shorter embryos and thinner seed coat had higher T_b than seeds with longer embryos and thicker seed coats. Thus, the thickness of the seed coat could be described as an adaptive trait. It is likely that thicker seed coats would protect the embryo from cooler temperatures than thinner seed coats (Lacey *et al.*, 1997). Therefore, lower temperatures are associated with an increase in the thickness of the seed coat similar to that observed at high altitude in *Chenopodium* (Dorne, 1981; Gutterman, 2000b) probably because of cooler temperatures.

In addition to temperature, precipitation controls the timing of germination in the wild. One general assumption is that germination occurs only if the monthly precipitation is >15 mm (Freas & Kemp, 1983;

Gutterman, 1993; Gutterman, 2000b). On this basis, I predicted the month in which the seeds will germinate. However, that assumption was not true for *B. rapa* subsp. *sylvestris* from Algeria, where the maximum monthly precipitation was not above 2 mm (*Appendix* Figure A5.1). The behaviour of *B. rapa* subsp. *sylvestris* from Algeria is similar to that of annual plants in an extreme desert climate (Gutterman, 1993) and it is one of the fastest CWR to germinate compare to the other *Brassica* CWRs. When water is regularly available, temperature becomes the major influence of germination timing.

In *Brassica* CWRs, θ_T and θ_H were positively correlated with mean precipitation in the month of germination, which is in agreement with the suggestion that seeds from drier environments might be adapted to germinate faster to avoid drought periods during seedling establishment (Evans & Etherington, 1990; Fenner & Thompson, 2004; Moles & Westoby, 2004a; Gardarin *et al.*, 2011) At the same time, T_b and Ψ_b were negatively correlated with mean precipitation in the month of germination which suggest that: 1) the T_b of the *Brassica* species is higher in drier environments; and 2) in drier environments the seeds are adapted to germinate rapidly in response to sporadic rainfall events that increase soil water potential and before the soil dries again. Additionally, these germination parameters were also correlated with the monthly mean precipitation of the maternal environment (seed collection site). According to the correlations found here, the germination of *Brassica* CWRs seems to be closely adapted to mean precipitation of the environment of seed collection site. On the other hand, the limitation of water during seed filling for the two research genotypes of *B. oleracea* had no significant effect on the majority of seed germination traits described in this chapter. This could suggest that the genetic diversity of the CWRs is due to a natural selection of the plants exposed to their natural environment and not resulting from a specific environment event (i.e., the limitation of water during seed filling).

5.3.1 Conclusions

Based on the thermal and hydro-time characteristics (thresholds and rates) and the conversion of germinated seeds into normal seedlings, *Brassica* CWRs appear better adapted than crops to future climate scenarios regarding water stresses. These adapted traits are somewhat predictable as the interspecific variation in germination functional traits (T_b, Ψ_b, θ_T and θ_H) correlate strongly to the climate at the origin of the seed lots, particularly to precipitation (the mean monthly precipitation and the mean precipitation of the month of germination). One possibility is that such data could be used to create an environmental envelope within which the minimum temperature and Ψ_b for *Brassica* species' seed germination can be considered in relation to climate change scenarios (Seal *et al.*, 2017). The findings of this chapter reinforce the need to characterise each seed lot, to provide a more precise prediction of seed vigour 'under a wide range of environments'.

6 CHAPTER 6: QUANTIFYING GERMINATION PERFORMANCE IN CROP WILD RELATIVES OF THE *HELIANTHUS* GENUS AND THE CROP *H. ANNUUS*

6.1 Introduction

The importance of CWRs in crop breeding programmes have increased (Dempewolf *et al.*, 2014; Seiler *et al.*, 2017), but some CWRs possess non-desirable traits for the crops such as seed dormancy. Therfore, a comprehensive description and characterisation of the CWRs seed functional traits is necessary to their successful use improving crops.

The *Helianthus* genus is wellknown because of its crop, *H. annuus* (sunflower) which is cultivated over the globe (Seiler & Gulya, 2015). *Helianthus* CWRs are spread in North America and possess great diversity which may serve as an important source of traits for biotic and abiotic stress tolerance for the improvement of commercial sunflower (Kane *et al.*, 2013; Seiler *et al.*, 2017). It is known that future warmer and drier environments will reduce seedling emergence of sunflower in addition to the appearance of diseases and fungi (Seiler *et al.*, 2017). The influence of such increases in temperature on the fatty acid composition of *H. annuus* oilseeds have also been explored (De Haro & Fernandez-Martinez, 1991; Linder, 2000). However, little is known of the natural variation in seed functional traits in *Helianthus* CWRs. This is particularly the case for perennial species, which often are affected by seed dormancy (Seiler *et al.*, 2017). In contrast, *H. annuus* is bred for fast and uniform seed germination under relatively optimal growing conditions, including irrigation. As the purpose of domestication is to create distinct, uniform and stable crops (Olsen & Gross, 2008), crop seed may have become inadvertently ill-adapted to future climate conditions.

Many *Helianthus* CWRs have seeds with deep physiological dormancy (Baskin & Baskin, 1998). This type of dormancy can arise from various tissues, including the embryo, seed coat and / or pericarp (Bewley, 1997; Baskin & Baskin, 1998; Weiss *et al.*, 2013). The combination of mechanical and biochemical changes in the pericarp affect the

development of seed dormancy in *Helianthus* species (Maeda & Ungaro, 1985), including prevention of radicle protrusion and interference with permeability to water (Seiler & Gulya, 2004). Pericarp thickness and distance from the radicle tip to the outside of the pericarp have also been suggested as modulators of germination in sunflower lines; shorter distances enabling faster germination in the crop (Weiss *et al.*, 2013) and this is hypothesised for all *Helianthus* seeds lots studied in this Chapter. Thus, both physical and physiological traits of the seeds can affect the germination response.

There have been some studies on the thermal control of germination in *Helianthus*, and lethal temperatures during germination and seedling emergence (40 to 45 ºC) in *H. annuus* cultivars have been identified (Gay *et al.*, 1991). Yoshida (1981) found at 40 ºC or higher temperatures, there was no cell division in the radicle tip of rice seeds. Therefore, it may be hypothesised that high temperatures have a deleterious effect on normal seedling development as cell division commences in the post-germination phase (Bewley, 1997). Moreover, low water potential could also restrain cell division in tomato seeds (de Castro *et al.*, 2000), and hence prevent normal seedling development. However, there are no studies to date that have quantified the thermal control of *Helianthus* CWRs germination, including lethal temperatures. Similarly, little is known about how low water potential affects seed germination and seedling growth in *Helianthus* CWRs.

The aim of this study was to identify and quantify the constraints to germination and subsequent conversion into normal seedlings of five *Helianthus* CWRs in comparison to the crop *H. annuus*. In five seed lots of *Helianthus* CWRs (Chapter 2, Table 2.3), of which four are perennial and suspected to have dormancy, and six crop genotypes (one commercial seed lot and five genotypes of *H. annuus* subjected to water treatments, Limagrain genotypes, Chapter 2, Table 2.4), various seed germination traits were quantified (dormancy, germination thresholds to temperature and water potential, and conversion of germinated seeds into seedlings). Furthermore, relationships between seed morphology, seed mass and oil content (described in Chapter 3) on seed germination traits are explored.

High concentration of oleic acid was correlated with faster germination as González Belo *et al.* (2014) found in sunflower genotypes. In this Chapter it was hypothesised that seeds with high oil content germinate faster than those with lower oil content. In addition, the impact of the fungal infection on seed germination and normal seedling growth in the Limagrain genotypes is observed. Finally, correlations between the environment of seed collection site and seed germination traits were investigated. The hypothesis that variability in seed germination traits may predispose *Helianthus* CWRs to cope better with temperature and water stress than Limagrain genotypes of *H. annuus* is discussed.

6.2 Materials and methods

6.2.1 Seed material

Five crop genotypes were provided by Limagrain (France). Each had a different genetic background, and varied in oil composition and in the earliness of their production (Chapter 2, Table 2.5). Plant growth and seed production were performed in the field at 16-32 °C (mean of the minimum and maximum temperatures during seed filling) in the south of Spain. Two water treatments were applied to plants of all five genotypes, normal irrigation and stopped irrigation during seed filling (see Chapter 2, section 2.2.3). In addition, another commercial seed lot of *H. annuus* was purchased from B&T World Seeds (hereafter referred to as the "commercial seed lot").

6.2.2 Seed dormancy

Fifteen seeds of each CWR were initially germinated at 20 °C to assess for the presence of dormancy. *H. argophyllus* reached 100 % of germination, however the four perennial wild seed lots did not reach 100 % (< 10 %). Dormancy was suspected and the germination experiments using several concentrations of gibberellic acid (GA_3) (Chapter 3, Figure 3.5) determined that 5 mM GA_3 with scarification was an effective dormancy breaking treatment of the perennial *Helianthus* CWRs (see Chapter 2 section 2.4.2).

For all perennial *Helianthus* CWRs, three replicates of 25 seeds were scarified and germinated at five constant temperatures from 10 to 35 °C ± 2 °C with and without 5 mM GA_3. Final germination was calculated on the basis of the total number of viable seeds sown (i.e., excluding empty and insect infested seeds based on a cut test). Since not all seed lots reached 100 % germination, the non-germinated but firm seeds were assumed viable and included to calculate the total viability.

6.2.3 Seed germination and normal seedlings

As described above, seeds of all perennial seed lots were scarified and germinated on 5 mM GA_3 except *H. argophyllus* seeds that germinated on distilled water without scarification. All crop seed lots were non-dormant and germinated on distilled water without scarification.

A germination experiment was assumed finished when the number of germinated seeds ceased for more than four weeks. The pericarp of non-germinated seeds was carefully removed at the end of the germination experiment. The embryos that were still firm were subjected to a vital staining test with 1 % 2,3,5-triphenyl tetrazolium chloride (Sigma Aldrich, UK) in darkness at 30 °C for 18 h. Embryos that were stained red or pink were considered viable (ISTA, 2003). The number of seeds that were empty or infested were noted and assumed to be dead. The level of germination (radicle emergence) and normal seedling production were each expressed as a percentage of the total number of viable seeds sown. Seedling normality was recorded following the definitions of ISTA (2017) as described in Chapter 2 section 2.4.1.

6.2.4 Fungal infection

Two hundred seeds of both treatments (normal and stopped irrigation) were subjected to x-ray to calculate the fungal infection level for all Limagrain genotypes as described in Chapter 3. The injured cotyledons had small holes, visible on the x-ray, where the fungus had established. Some of these “spotted” seeds, assumed to be infected, were opened to visually confirm the fungal infection of the embryo as described in Chapter 3. Furthermore, seeds of the genotype B (of two water treatments) that did

not show signs of fungal infection (non-infected seeds) were separated from the mixed seeds (control, infected and non-infected seeds in the proportion described in Chapter 3, Table 3.4) based on x-ray results. For non-infected seeds and mixed seeds three replicates of 25 seeds each were sown at 30 °C, 25 °C and 20 °C to compare seed germination performance and seedling normality. These temperatures were chosen to be around the optimal temperature for germination rate as determined by thermal time modelling (below, section 6.3.2).

6.2.5 Data analysis

Sigmoidal curves of seed germination were calculated using Origin 9.1 software. To compare the mean seed germination (%) and normal seedling (%), the mean percentages were arcsine transformed prior to the analysis of the data (see Chapter 2 section 2.8). The mean values were compared between populations using the Fisher test (F-test) and student's t-test (t-test). Correlations between seed traits and the maternal environment of CWRs were plotted in a scatter matrix and then linear regressions were performed.

6.3 Results

6.3.1 Dormancy of *Helianthus* CWRs

Although non-scarified seeds of *H. argophyllus* reached 100 % germination, non-scarified seeds of the other four CWRs did not exceed 10 % germination (data not shown). Scarification increased the germination of the four dormant seed lots with values of up to 36 % obtained when imbibed with water (Chapter 3, Table 3.3). Seed scarification was used in combination with GA_3 in the dormant seed lots improving significantly ($P < 0.05$) seed germination compared with the germination experiments using only GA_3 (Chapter 3, Table 3.3). Thus, for all further germination experiments, seed scarification was combined with 5 mM GA_3 to break dormancy in all *Helianthus* CWRs except *H. argophyllus* which was germinated without any pre- treatment.

6.3.2 Germination trait parameters

Seed germination traits

Seed germination of five *Helianthus* CWRs had base temperatures (T_b) differing by 12 °C, from 1.0 °C (*H. argophyllus*) to 13.3 °C (*H. angustifolius*, N. Carolina, Figure 6.1, Table 6.1). The estimated ceiling temperature (T_c) range for germination progress of these seed lots was from 29.3 °C to more than 35 °C. T_c could not be estimated for *H. glaucophyllus* seeds as 35 °C, the highest incubation temperature used due to number of seeds available, was still in the sub-optimal temperature range for seed germination according to the thermal time model (Figure 6.1 A). The optimal temperature (T_o) was therefore not calculated for *H. glaucophyllus*. For the other wild *Helianthus* seed lots, T_o ranged widely from 14.9 °C to 30.6 °C. Amongst the *H. annuus* Limagrain genotypes and the commercial seed lot T_b values varied less than the CWRs, varying between 3.3 °C and 5.2 °C.

Thermal time (θ_T) values, for the sub-optimal temperature range, differed more than three-fold amongst *Helianthus* CWRs, from 1350.1 °Ch for *H. angustifolius* Texas to 4552.3 °Ch for *H. glaucophyllus* (Table 6.1). The crop seed lots had shorter θ_T (i.e., faster germination) than the CWRs, from 590.3 °Ch for genotype C 'stopped irrigation' to 829.9 °Ch for genotype B 'normal irrigation'. Within the temperature range used in this study, it was possible to estimate T_c and θ_{Tsupra} for most of the CWRs. However, it was not possible to estimate θ_{Tsupra} for the crop genotype seeds as all but one of the temperatures were in the sub-optimal temperature range. On the other hand, due to the availability of seeds, the seeds of the commercial seed lot were imbibed at three temperatures (38, 40 and 42 °C) in the supra-optimal range (Figure 6.1 I). Thus, T_c and θ_{Tsupra} were estimated in the commercial seed lot, 45.3 °C and 172.3 °Ch respectively (Table 6.1).

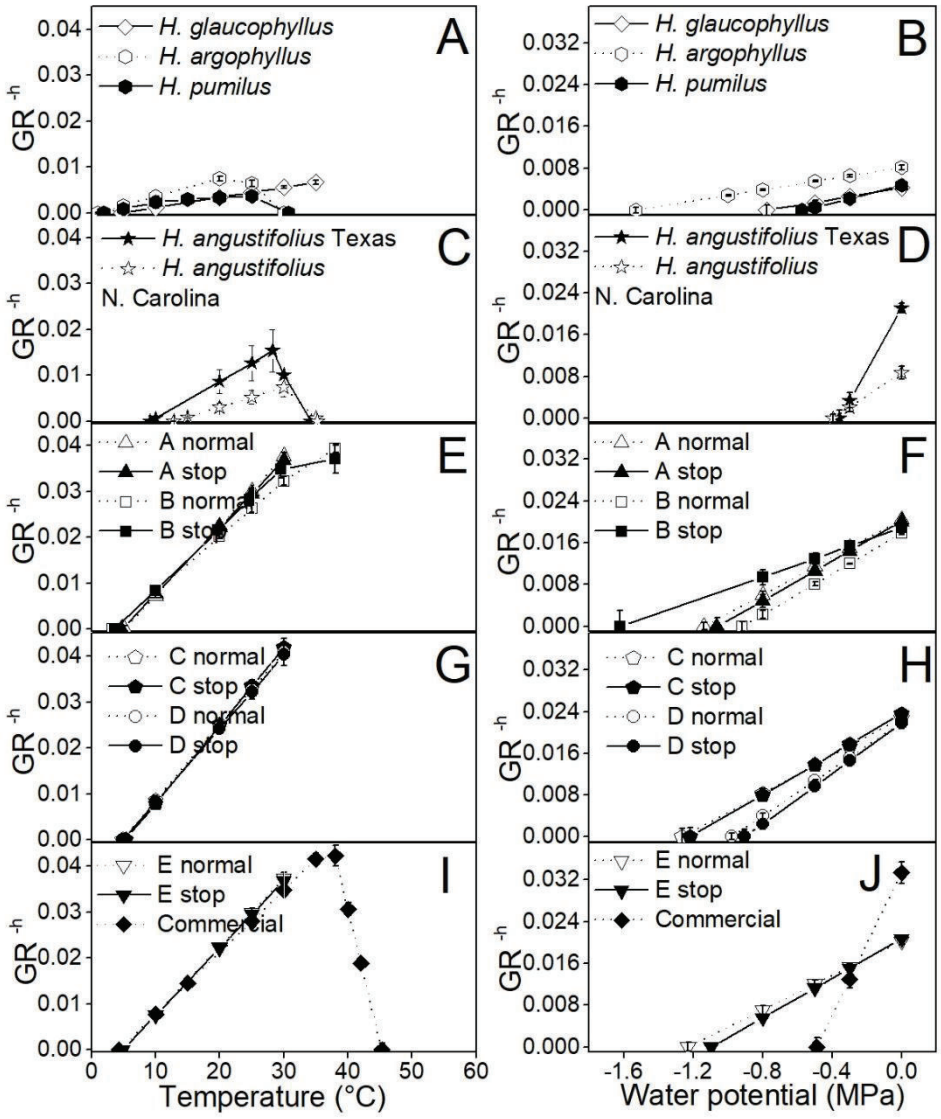


Figure 6.1 Thermal and hydro time model description for the *Helianthus* genus. The germination rate (GR) was plotted against temperature (A, C, E, G and I) to estimate the cardinal temperatures, base temperature (T_b), optimal (T_o) and ceiling (T_c) temperature. The GR was also plotted against water potential to estimate the base water potential (B, D, F, H and J). Five CWRs (A-D), five Limagrain genotypes of *H. annuus* (E-J) and one commercial seed lot (I and J). Limagrain genotypes A, B, C, D and E were subjected to two water treatments, normal and stopped irrigation (E-J). The seeds were germinated under a range of sub- and supra-optimal temperatures between 5 to 42 °C and on PEG solutions at 0, -0.3, -0.5, -0.8 and -1.0 MPa at one constant temperature (between 15 and 30 °C). The regression lines were calculated from repeated probit analysis estimations and the error bars are the SD of three replicates for each treatment in CWRs and four replicates for the crops. For most of the data, the error bars are smaller than the symbols (A, B, D, F, G, H, I and J).

The calculation of T_o in the CWRs seeds ensured that the water potential conditions were performed at temperatures below the supra-optimal range. Lower water potential conditions reduced the final germination percentage and slowed the GR. Ψ_b differed in the CWRs seeds from -0.4 MPa (*H. angustifolius* Texas) to -1.53 MPa (*H. argophyllus*, Figure 6.1B, Table 6.1). In general, the crop genotypes were less variable than the CWRs (Table 6.1). The seeds of *H. annuus* had lower Ψ_b than the CWRs, except for the *H. argophyllus* seed lot. The range of θ_H in the CWRs fell into two groups: *H. angustifolius* (both seed lots) with shorter θ_H (from 17.3 to 45.3 MPah); and the other three species (*H. glaucophyllus, H. argophyllus* and *H. pumilus*) with larger θ_H from 126.0 to 188.3 MPah. The same groups were found for θ_{HT}, where seeds of *H. angustifolius* had the lowest value within CWRs (597 and 1041 °CMPah for North Carolina and Texas seed lots, respectively). The range of θ_H values for crop genotype seeds was within the range of the CWRs (between 41.7 to 86.3 MPah, Table 6.1). The seeds of the commercial seed lot showed the lowest θ_H and $\theta_{HT,}$ 14.8 MPah and 421.5 °CMPah respectively within the crop seed lots.

The tetrazolium test on the non-germinated seeds of the dormant *Helianthus* CWRs was performed on firm embryos from the germination experiments at low water potentials. The test showed 25 % of the non-germinated seeds had embryos that were still viable (fully stained in red-pink). The embryos of non-germinated seeds of the seed lots of *H. annuus* were mouldy and therefore performing a tetrazolium test was not possible.

Table 6.1 Seed germination threshold model parameters of the *Helianthus* genus, five *Helianthus* CWRs and 11 crop seed lots of *H. annuus*. Cardinal temperatures are defined as base temperature (T_b), ceiling temperature (T_c) and optimal temperature (T_o) for the progression of germination. The thermal time models relate to the sub-optimal range (θ_T) and supra-optimal range of temperatures (θ_{Tsupra}). Hydro time modelling relates to the base water potential (Ψ_b) threshold and hydro time (θ_H) for the rate of germination progress. The hydrothermal time (θ_{HT}) was calculated using the mean of the other parameters using Equation 2.9 from Chapter 2. Different letters denote significant differences between the CWRs or, separately, between the crop seed lots. # There was a plateau between 14.9 and 25 °C To

CWRs	T_b (°C)	θ_T (°Ch)	T_c (°C)	θ_{Tsupra} (°Ch)	T_o (°C)	Ψ_b (MPa)	θ_H (MPah)	θ_{HT} (°CMPah)
H. glaucophyllus	4.7 d	4552.3 a	> 35	-	-	-0.78 b	185.0 a	3779
H. angustifolius N. Carolina	13.3 a	2901.0 bc	35.2 a	300.0 b	30.6	-0.40 a	17.3 b	597
H. angustifolius Texas	9.2 c	1350.1 b	34.2 a	416.7 b	28.3	-0.36 a	45.3 b	1041
H. argophyllus	1.0 b	2549.7 bc	29.3 a	540.0 b	24.4	-1.53 c	188.3 a	4331
H. pumilus	1.9 b	3425.0 ac	30.7 a	16250.0 a	14.9 – 25#	-0.58 b	126.0 a	2388
Crop *H. annuus*								
A normal irrigation	5.2 e	659.4 de	-	-	-	-1.14 d	55.7 c	1233
A stopped irrigation	4.7 ef	689.3 de	-	-	-	-1.06 d	53.0 c	1293
B normal irrigation	3.3 g	829.9 f	-	-	-	-0.92 d	51.5 c	1030
B stopped irrigation	3.6 gf	760.8 df	-	-	-	-1.62 e	86.3 d	1875
C normal irrigation	4.8 ef	601.0 e	-	-	-	-1.27 d	55.3 c	1172
C stopped irrigation	5.3 e	590.3 e	-	-	-	-1.22 d	51.7 c	1087
D normal irrigation	4.8 ef	616.4 e	-	-	-	-0.98 d	44.7 c	968
D stopped irrigation	4.8 ef	628.0 e	-	-	-	-0.91 d	41.7 c	874
E normal irrigation	4.8 ef	676.2 de	-	-	-	-1.23 d	60.7 c	1304
E stopped irrigation	4.8 ef	685.4 de	-	-	-	-1.10 d	53.0 c	1132
Commercial seed lot	4.3 efg	741.6 df	45.3	172.3	37.2	-0.49 f	14.8 e	422

Conversion of germinated seeds into normal seedlings

All germinated seeds in the CWRs converted into normal seedlings, with the exception of *H. pumilus* at 20 °C and *H. glaucophyllus* and *H. pumilus* at 10 ℃ (Figure 6.2); where the percentage normal seedlings were significantly lower than percentage seed germination. In contrast, the percentage normal seedlings were lower than seed germination at most of the temperatures for the crop genotypes (Figure 6.3 and 6.4). The most dramatic and significant reduction in conversion to normal seedlings were observed at high temperatures (30 and 35 °C), for both crop production environments (normal and stopped irrigation).

Seedling normality was also recorded at several water potentials. The percentage normal seedlings did not differ from seed germination (radicle emergence) in the CWRs with the following exceptions: *H. glaucophyllus*, where only half of the germinated seeds at -0.5 MPa were converted into normal seedlings (Figure 6.2), and *H. pumilus* at 0 MPa, where the normal seedlings were 10 % lower than the germinated seeds. In contrast, the percentage of germinated seeds was significantly higher than the percentage normal seedlings (Figure 6.3 and 6.4) for all Limagrain genotypes under most of the water potential conditions except for genotype C (stopped irrigation) and both treatments of genotype E.

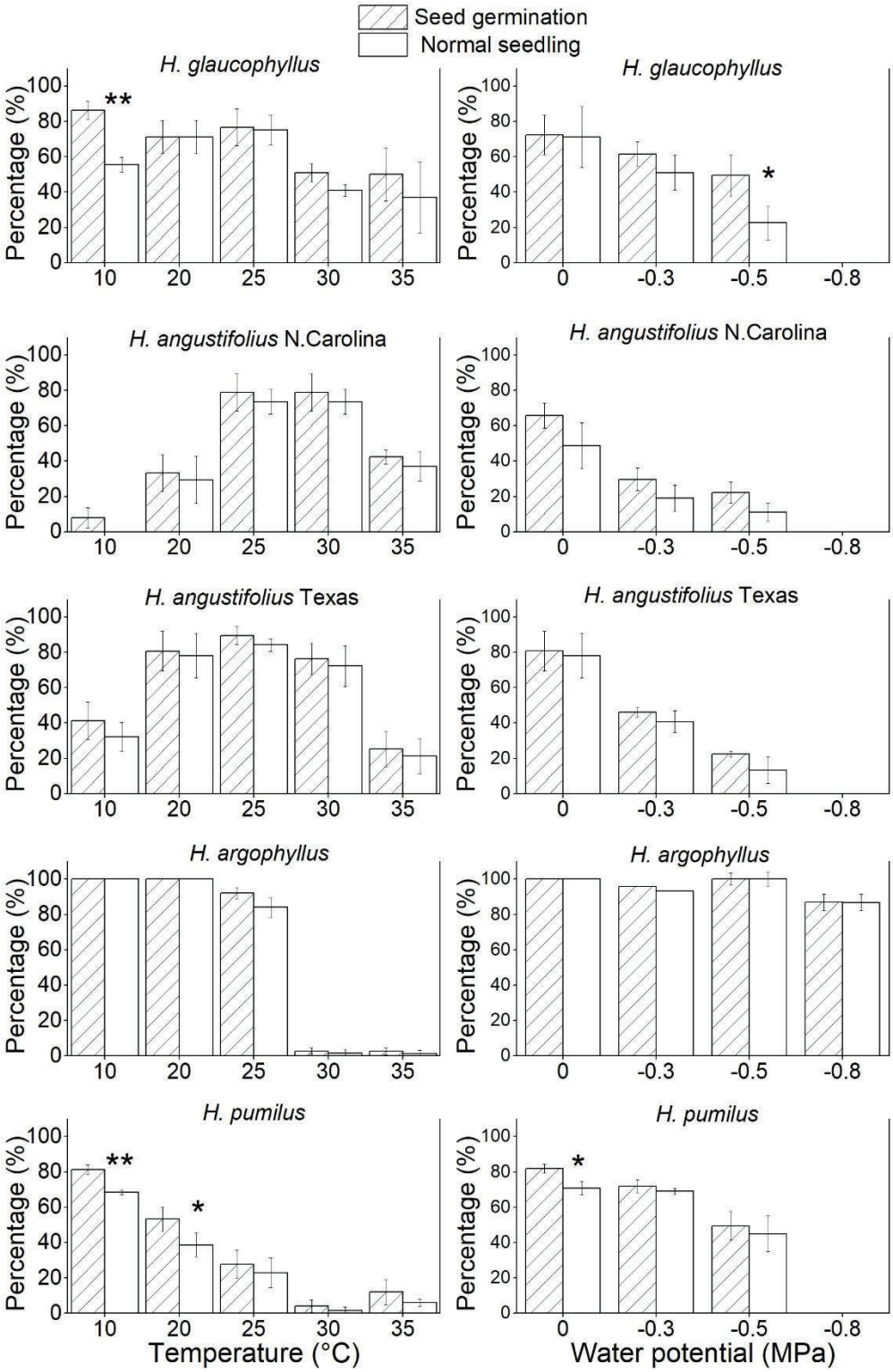


Figure 6.2 Conversion of germinated seeds into normal seedlings of *Helianthus* CWRs. Comparison of seed germination (radicle emergence, striped bars) and normal seedling production (open bars) in five *Helianthus* CWRs subjected to a range of temperatures (left) and water potentials (right). The bars are the mean of three replicates with the error bars showing the SD. Significant differences between seed germination and normal seedling percentages at each condition are indicated by asterisks (* $P < 0.05$ and ** $P < 0.01$).

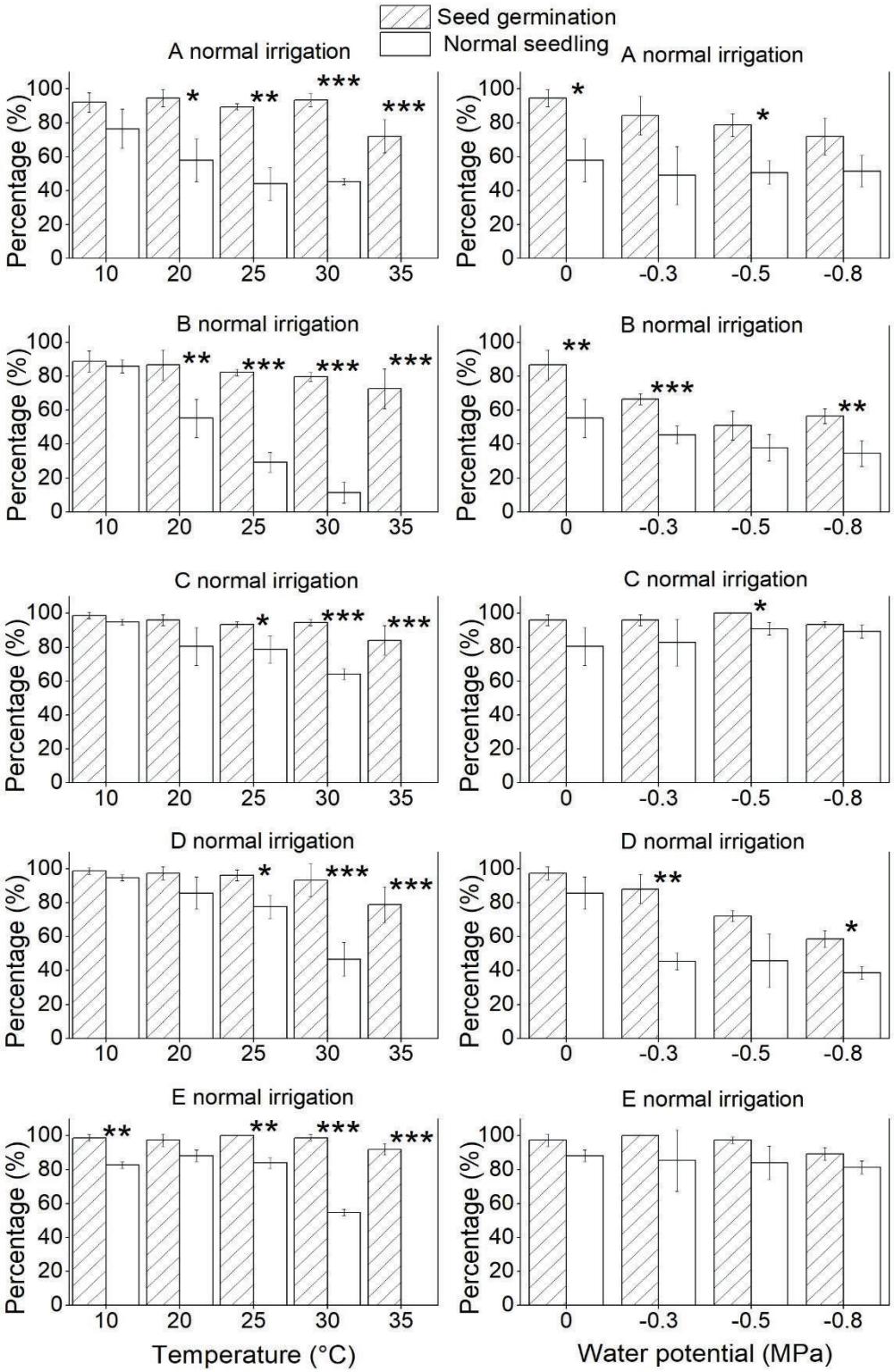


Figure 6.3 Conversion of germinated seeds into normal seedlings of Limagrain genotypes of *Helianthus annuus* normal irrigation treatments during seed filling. Seed germination (striped bars) and normal seedling (open bars) percentages subjected to a range of temperatures (left) and water potentials (right) of five *Helianthus* crop genotypes (A-E). The bars are the mean of four replicates with the error bars showing the SD. Significant differences between seed germination and normal seedling percentages at each condition are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

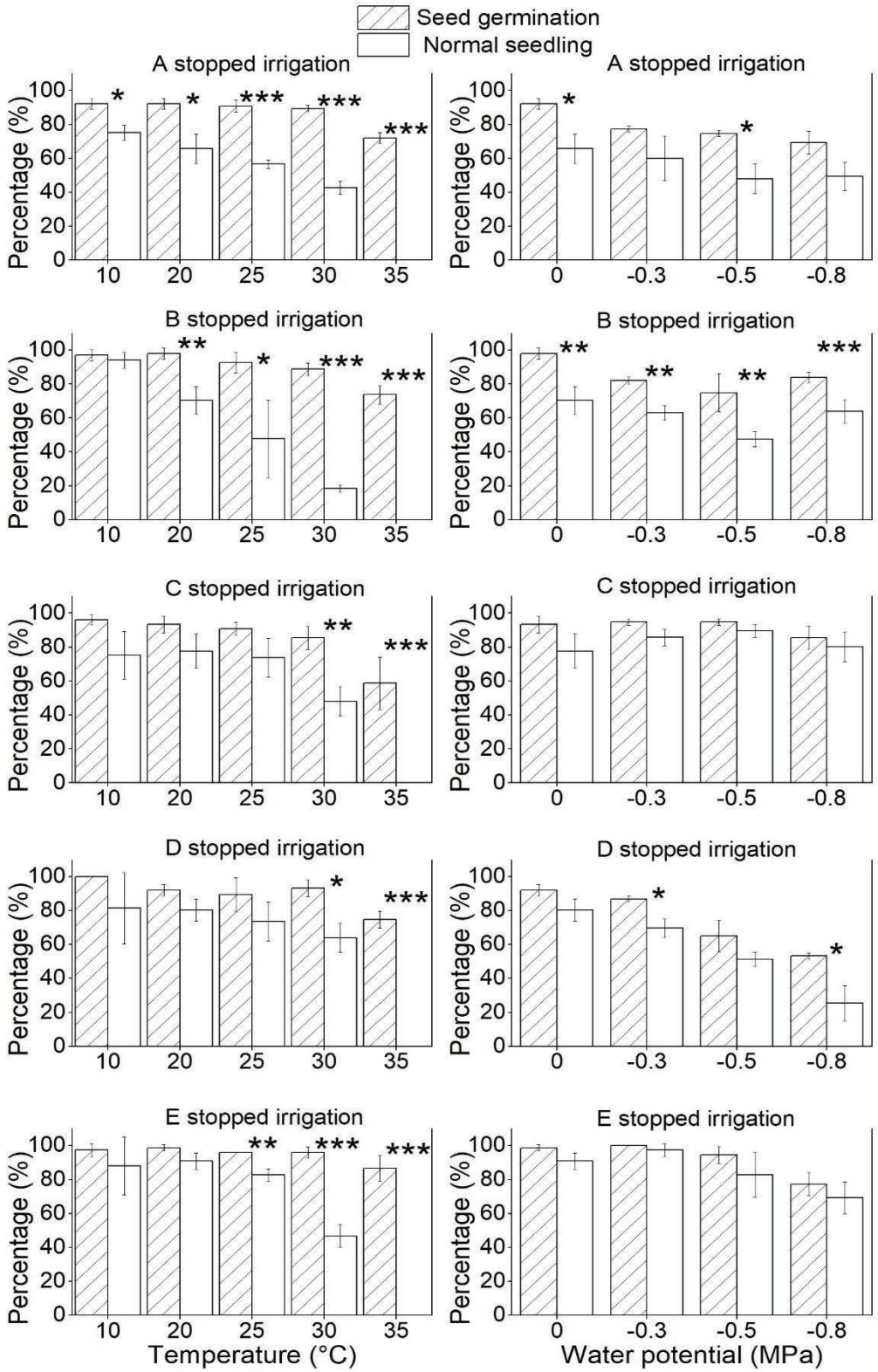


Figure 6.4 Conversion of germinated seeds into normal seedlings of Limagrain genotypes of *Helianthus annuus* stopped irrigation treatments during seed filling. Seed germination (striped bars) and normal seedling (open bars) percentages subjected to a range of temperatures (left) and water potentials (right) in five *Helianthus* crop genotypes (A-E). The bars are the mean of four replicates with the error bars showing the SD. Significant differences between seed germination and normal seedling percentages at each condition are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

The level of fungal infection was estimated from x-ray images in the Limagrain genotype seed lots (Chapter 3, Table 3.4). More than half of the seeds of genotype B were infected in both irrigation treatments (normal and stopped seed lots).

A comparison was made between seed germination and normal seedling percentages of non-infected seeds and mixed seeds (infected and non-infected seeds in the proportion found in Chapter 3, Table 3.4) for the crop genotype B (Table 6.2). No significant differences were found between non-infected seeds and mixed seeds in germination percentage ($P > 0.05$). Percentage normal seedlings was higher for the non-infected seeds compared to the mixed seeds at 20 °C and 25 °C for both plant production treatments ($P < 0.01$). However, at 30 °C in both populations (normal and stopped irrigation) there was a significant decrease in the conversion into normal seedlings in the non-infected seeds. Thus, the decrease of normal seedlings was not "only" because of the fungal infection but also the temperature, especially at 30 °C. (*Appendix* Figure A6.1).

Table 6.2 Conversion of germinated seeds into normal seedlings of non-infected and mixed seeds in *Helianthus annuus* genotype B. Seed germination (G %) and normal seedling (S %) production were compared at three temperatures (T; 20, 25 and 30 °C) of *H. annuus* (crop genotype B) from seeds harvested from plants subjected to two treatments (normal and stopped irrigation). Non-infected (NInf) seeds were separated on the basis of x-ray images and compared with mixed seeds (infected and non-infected seeds, randomly selected). The asterisks represent significant differences ($P < 0.05$) between normal seedlings and seed germination within each temperature and treatment.

T (°C)	Normal NInf		Normal mixed		Stopped NInf		Stopped mixed	
	G %	S %	G %	S %	G %	S %	G %	S %
20	92.0	89.3	86.6	55.3*	100	100	98.0	70.3*
25	88.0	77.3*	82.3	29.3*	100	82.7*	92.7	47.6*
30	89.3	28.0*	79.6	11.3*	96	64*	88.7	18.4*

6.3.3 Correlations between seed traits

The seed germination traits of CWRs and *H. annuus* seed lots were compared using linear regression models. A positive correlation was found between Ψ_b and T_b ($P < 0.001$; $r = 0.54$, Figure 6.5). Moreover, the mean percentage of oil content was negatively correlated with θ_T (Figure 6.6 A) and Ψ_b (Figure 6.6 B). Therefore, seeds with higher oil content need to accumulate less θ_T to germinate and possess lower Ψ_b.

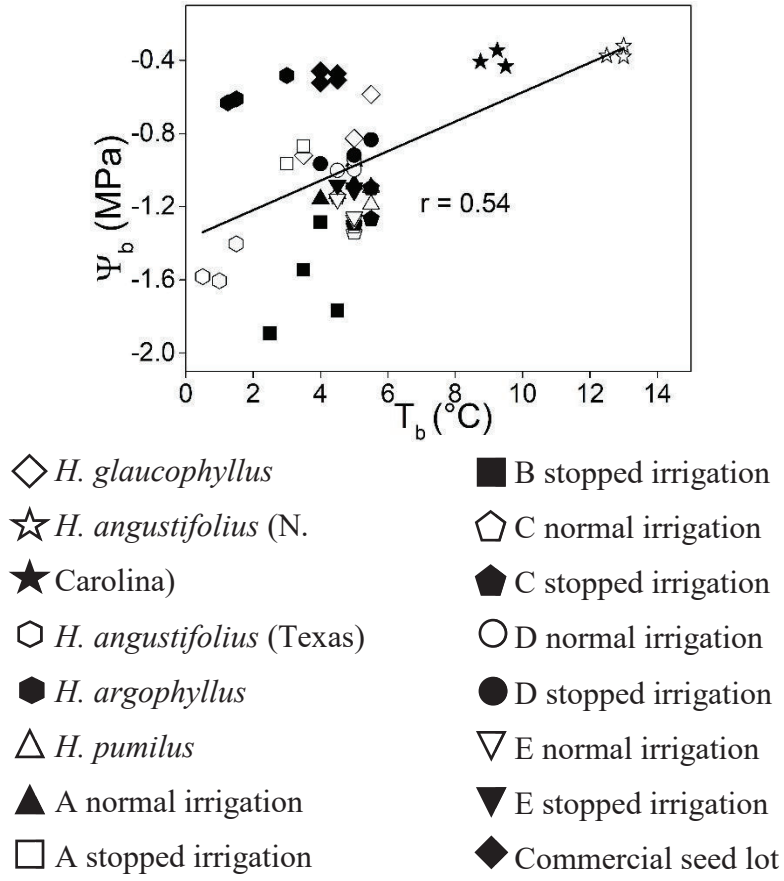


Figure 6.5 Significant correlation between base water potential (Ψ_b) and base temperature (T_b) of 16 *Helianthus* seed lots (five CWRs and six crop genotypes) ($P < 0.001$). Each point represents a replicate within each seed lot. DF = 46

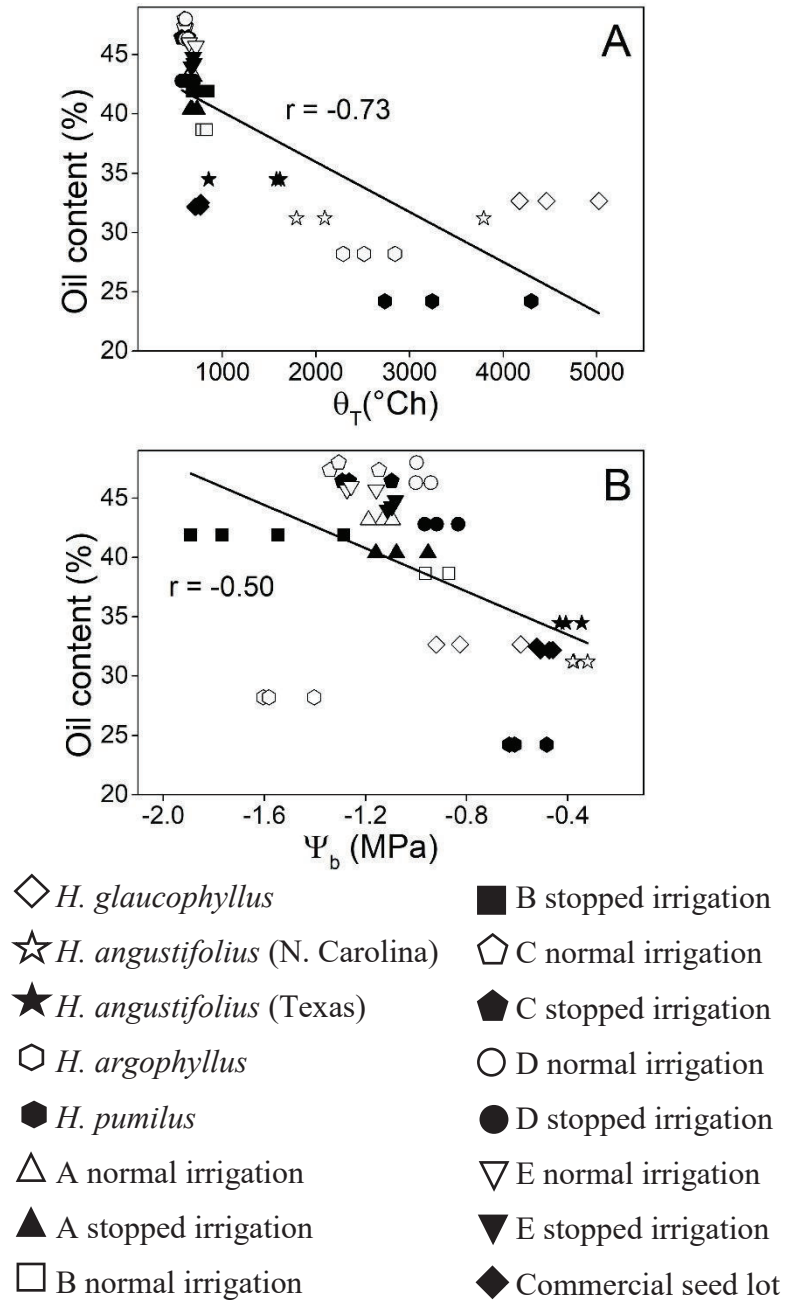


Figure 6.6 Correlations between oil content and seed germination traits of 16 *Helianthus* seed lots (five CWRs and six crop genotypes). Mean seed oil content (%) was negatively correlated with: A) thermal time (θ_T, $P < 0.001$, $DF = 46$); and B) base water potential (Ψ_b, $P < 0.001$, $DF = 46$). Each point represents a replicate of each seed lot.

With regard to the seed characterisation described in Chapter 3, θ_T was positively correlated with the value of the pericarp thickness (i.e., pericarp thickness divided by the embryo length) for all *Helianthus* CWRs and crop seed lots (Figure 6.7). Thus, seeds with thicker pericarps (in relation to the embryo length) need to accumulate more θ_T to germinate.

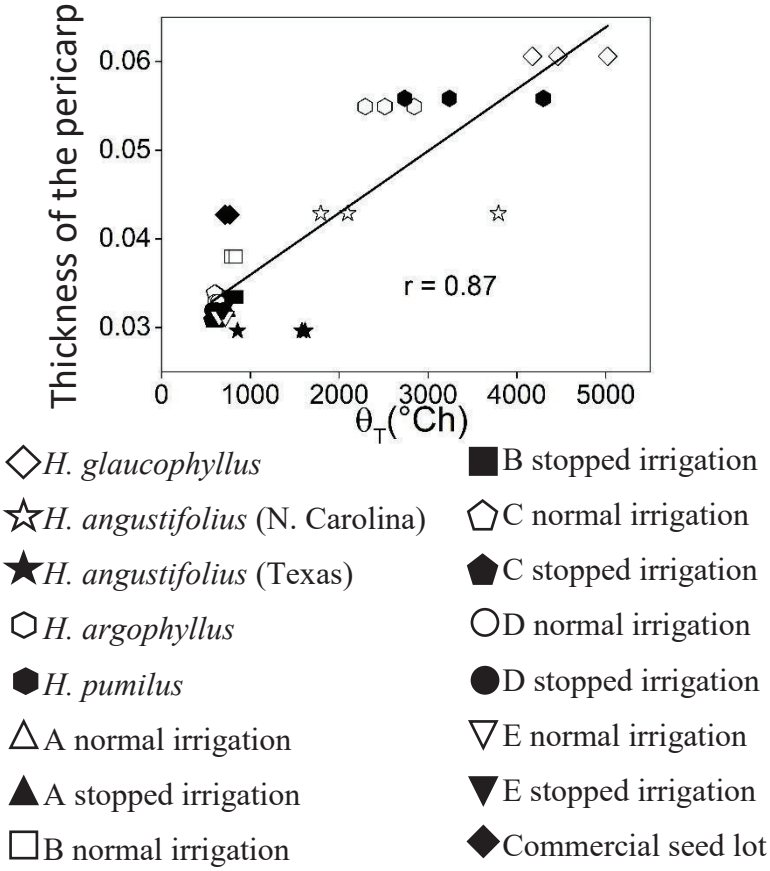


Figure 6.7 Significant correlation between the pericarp thickness, divided by the embryo length, and thermal time (θ_T) of 16 *Helianthus* seed lots (five CWRs and six crop genotypes) ($P < 0.001$). Each point represents a replicate of each seed lot. DF = 46

6.3.4 Correlations between the environment of origin and seed functional traits

The dependencies of seed germination traits on the environment of the seed collection site were assessed for the five *Helianthus* CWRs. From all the correlations analysed between seed traits of CWRs and their environment, the only significant correlation was identified between the historical annual mean temperature (minimum, mean and maximum) and

θ_T (Figure 6.8). Thus, CWRs seeds from warmer environments had faster germination (i.e. shorter θ_T). Finally, the altitude was not correlated ($P > 0.05$) with any germination parameter; and seed functional traits did not correlate with the temperatures nor precipitation of the predicted month of germination (*Appendix* Figure A6.2) or at other times of the year when plant developmental events such as flowering would have occurred.

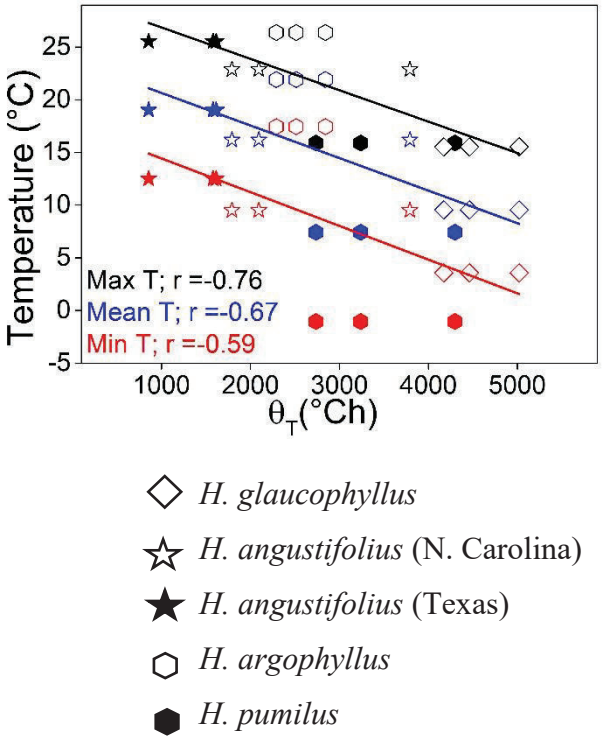


Figure 6.8 Significant correlation between the annual mean temperature and the thermal time (θ_T) of five *Helianthus* CWRs ($P < 0.05$). Annual mean maximum is represented by black symbols and line, mean temperature is represented by blue symbols and line and minimum temperature is represented by red symbols and line. Each point represents a replicate of each seed lot. DF = 13

6.4 Discussion

A major constraint to the managed use of *Helianthus* CWRs in revegetation programmes is the presence of seed dormancy. Both the seed coat and pericarp have been shown in some species to physically restrain the radicle (Esashi & Leopold, 1968), and/or prevent water from reaching the embryo, gaseous exchange, or blocking the escape of inhibitors from the embryo (Baskin & Baskin, 1998; Finch-Savage & Leubner-Metzger, 2006; Gosling, 2006; Rathjen *et al.*, 2009). Although the seeds of the

Helianthus CWRs did not have impermeable tissues (seeds gained weight during the imbibition process without scarification, *Appendix* Table A3.2), the pericarp did serve as a partial physical barrier, as radicle emergence in the perennial species improved with scarification (Chapter 3, Table 3.3). One adaptive value of having relatively thicker seed covering structures is to serve as protection against predation and is associated with increased persistence and decreased mortality in the soil seed bank (Fenner, 1983; Blate *et al.*, 1998; Gardarin *et al.*, 2010; Schutte *et al.*, 2014).

To facilitate germination in *Helianthus* CWRs, seed scarification of the pericarp at the embryonic axis end in addition to 5Mm GA_3 was insufficient to ensure high levels of germination. This indicated the presence of both a partial mechanical barrier to germination and deep physiological dormancy (Baskin & Baskin, 1998).

The diversity of germination performance in terms of thermal and hydro traits for *Helianthus* seeds (CWRs and crops) has been described in this chapter. Seed germination trait analysis indicated a narrower range of T_b in the *H. annuus* seed lots compared with the CWRs (1.0 to 13.3 °C). Moreover, the T_b of the crop seed lots was similar irrespective of the different oleic acid content, contrary to the findings of González Belo *et al.* (2014). In this study, all crop genotypes from Limagrain were grown in the same field conditions in the south of Spain during the same year, thus it is possible that T_b did not differ because the Limagrain genotypes grew in the same environment. Supporting this notion is the observation that the CWRs studied, which grow in environments varying by > 10 °C annual mean temperature, are well adapted to their environments and, consequently, are likely to need a much wider range of estimated T_b.

Once above the threshold for germination (T_b), the *Helianthus* CWRs required longer thermal times (θ_T) for germination than *H. annuus* seed lots. The slow germination in the seed lots of CWRs highlights an impact of crop selection for seed vigour (Finch-Savage & Bassel, 2015). Faster germination (shorter θ_T) is associated also with high oil content across all *Helianthus* species, rather than limited to crop genotypes with high concentration of oleic acid as suggested by González Belo *et al.* (2014). Seeds with high oil content in the *Brassica* genus were positively

correlated with higher θ_H (slower germination under low water potentials, Chapter 5 Figure 5.3 C). Across genera, it is not possible to make such clear correlations between oil content and germination speed on a thermal time basis. For example, oily species are not always the fastest to germinate, and non-oily seeds of grasses have similar thermal times to the oily seeds of aromatic and medicinal plants (Dürr *et al.*, 2015). Small amounts of oil in the axis or endosperm may be degraded to provide some energy to the germinating seeds; however, the main storage oils in the cotyledons are not mobilised until post germination (Bewley *et al.*, 2013; Munz *et al.*, 2017). Seeds with relatively thicker pericarps (divided by the embryo length) required more time to germinate (longer θ_T) than those with relatively thinner pericarps. This relationship found for the 16 *Helianthus* seed lots (CWRs and crops) agrees with that suggested by Weiss *et al.* (2013) for *H. annuus* seed lots (cultivar, wild type and hybrids). In this chapter, seeds of CWRs possessed relatively thicker pericarps, when the value was divided by the embryo length, and longer θ_T (slower germination) was required for the germination process compared to the crop seed lots. Thus, pericarp relative thickness could be suggested as a regulator of germination in the *Helianthus* genus or it may just be a negative consequence of a separate benefit of thicker pericarps, such as a better protection barrier for the embryos which might provide longer life-span in the seedbed (Mohamed-Yasseen *et al.*, 1994). Moreover, seeds with high oil content possessed lower Ψ_b for all the seed lots (CWRs and crops). The relationship between oil content and moisture content has been studied in many biological systems (see Fontana & Campbell, 2007). Without fail, oily seeds (e.g., rapeseed) have lower water contents than non-oily seeds (e.g., barley) under any equivalent water activity or relative humidity. Therefore, sunflower seeds with increased oil content might be expected to germinate in equilibrium with lower water potentials, as observed in Figure 6.6 B. Interestingly, this general effect is observed amongst all *Helianthus* seeds tested here, and is not restricted to the crop genotypes, suggesting close dependency of seed water relations on oil content.

Radicle emergence is the end of the germination phase but the conversion of germinated seeds into normal seedlings is another critical step to becoming an adult plant. For this reason, seedling normality as well as seed germination (i.e., radicle emergence) is often the preferred means of assessing seed vigour in the seed trade (ISTA, 2017). In this chapter, wild *Helianthus* seed lots were able to produce normal seedlings under most of the conditions tested, whereas the crop genotypes of *H. annuus* had low or no normal seedling growth at higher temperatures and low water potentials (i.e. away from optimal conditions used in seed testing). Even though some fungal infection affected seed germination and normal seedling production, the conversion of non-infected germinated seeds to normal seedlings at high temperatures (i.e. 30 °C) also decreased significantly for crop genotype B (Table 6.2). Moreover, this significant decrease was observed in the Limagrain genotypes with low infection levels. For example, genotypes C and E at 30 and 35 °C.

It is worthy of note that previous chapters identified the lack of conversion of germinated seeds into normal seedlings in seed lots of *Hordeum* and *Brassica* at high temperatures. Similarly, an overestimation of seed viability and performance post-storage was shown in species from the Salicaceae family, with normal seedling production systematically lower than radicle emergence (Popova *et al.*, 2013; Ballesteros & Pence, 2017). Although Gay *et al.* (1991) identified the range of 40 to 45 °C as lethal to seed germination and seedling emergence in sunflower, the data presented in this chapter suggests that some sunflower genotypes can struggle to produce normal seedlings at 35 °C. Thus, estimates of normal seedlings should be given greater emphasis when considering seed performance under high temperature or drought stress in the seed bed.

As for 27 European weed species, including seven species in the Asteraceae (Gardarin *et al.*, 2010), there is a positive correlation between T_b and Ψ_b in seeds of *Helianthus* species. This suggests that seeds with a higher temperature threshold for the onset of germination also tend to require higher water potentials (i.e. relatively wet conditions) for germination. When germinated under a series of different water potentials, the seeds of CWRs generally had higher Ψ_b than the crop genotypes seeds.

Increasing concentrations of GA_{4+7} are known to lower seed Ψ_b, probably due to dormancy release (Ni & Bradford, 1993; Alvarado & Bradford, 2005). This suggests that the poor performance of *Helianthus* CWRs seed lots at lower Ψ could be because a proportion of the seed population was still dormant. In agreement with this perspective, non-dormant seeds of *H. argophyllus* have Ψ_b similar to those of the crop genotypes. In *Arabidopsis*, many of the most highly expressed genes in dormant states are stress-related even when abiotic stress is absent (Cadman *et al.*, 2006). It seems likely, therefore, that the combined 'stress' of some residual dormancy and reduced water potentials in *Helianthus* CWRs contributed to higher Ψ_b in these seed lots. Non-germinated seeds of *H. argophyllus* at the end of the germination test stained positively for tetrazolium, indicating viable seeds. In contrast, the other more dormant seed lots of *Helianthus* CWRs had a weaker staining reaction. It is known that seeds with deep dormancy might not stain brightly with tetrazolium (Peters, 2000). Therefore, future studies should explore ways to improve dormancy alleviation (scarification and GA_3) in *Helianthus* CWRs to ensure germination greater than *c.* 80% achieved in this study.

Several studies have revealed the close relationship between seed germination traits and the environment in which the seeds were produced (Harel *et al.*, 2011; Porceddu *et al.*, 2013; Galíndez *et al.*, 2017; Seal *et al.*, 2017). For the seeds of the *Helianthus* CWRs studied here, the only correlation between any environmental factor and the model parameters was the historical annual mean temperature positively correlated with the θ_T. Warm temperatures were associated with longer distances between the radicle and the pericarp (see Chapter 3, Figure 3.9). However, this may not be a disadvantage for the seeds in terms of germination since seeds from warm temperatures also germinated faster (short θ_T). It has been described in several species that seeds from plants growing at high temperatures had faster dormancy loss rate than those which mother plants are growing at lower temperatures (Fenner, 1991). The interplay between environment and plasticity of seed functional traits in *Helianthus* CWRs appears intricate. Here, it involves pericarp features as well as θ_T, which in turn is correlated with oil content. More widely, seeds with low T_b tend to

germinate slowly on a thermal time basis (i.e., longer θ_T). Thus, this might reduce the chance of a whole wild population of seedlings being eradicated by a late frost (*Brassica* seeds in Chapter 5, Trudgill *et al.*, 2000; Dürr *et al.*, 2015; Seal *et al.*, 2017). These illustrate complex responses to the environment that are manifested as subtle changes in germination traits that are clearly of adaptive value.

In conclusion, *Helianthus* CWRs appear better equipped than crop genotypes of *H. annuus* to cope with the higher temperatures and drought conditions projected for North America and Europe as a result of global warming, (IPCC, 2013). Moreover, predictable differences in seed functional traits with the environment of the seed collection site could be used to inform the choice of other *Helianthus* species for revegetation programmes. However, existing constraints to their use, particularly for perennial species (Atlagić & Terzić, 2016), reinforce the need for better understanding of seed dormancy in *Helianthus* CWRs and the development of better dormancy alleviation treatments.

7 CHAPTER 7: SEED LONGEVITY OF TWO POPULATIONS OF THE CROP GENOTYPE B OF *H. ANNUUS*

7.1 Introduction

There are *c.*7.4 million plant accessions conserved *ex situ* in the world, the vast majority of which are stored as seed lots. In the last 20 years almost two million seed accessions were stored (FAO, 2010). Seed conservation, among others, is important for the preservation of plant diversity, including that of CWRs. Ideally, the species should be conserved *in situ* in nature. However, due to the environmental fluctuations and extreme weather events, some species have suffered genetic erosion or extinction (Hawkes *et al.*, 2012). *Ex situ* seed conservation, therefore, seeks to ensure the survival of species and to complement *in situ* conservation efforts in protected areas via species and habitat restoration programs (Cochrane *et al.*, 2007).

It is widely known that the main parameters that can be controlled to increase the life-span or longevity of the seeds preserved *ex situ* are storage temperature and humidity. The storage behaviour of seeds that have improved longevity on drying and cooling is called orthodox (Roberts, 1973). Most of the world's seed-bearing plants (92 %) are predicted to produce seeds that are orthodox (Wyse & Dickie, 2017).

Seed longevity is the capacity of a seed lot to maintain viability over time with the survival time dependent on the specific storage condition (environment). Understanding how to improve seed storage and seed longevity is important for the maintenance and development of seed banks. In addition, it is also advantageous for seed companies or farmers that store seeds for shorter periods of time, i.e. one year. As a general rule, seed longevity doubles with a 1 % decrease in seed moisture content (MC) or 5 °C decrease in the storage temperature for orthodox seeds (Harrington, 1972). However, there are some limits to this response (Roberts, 1986; Vertucci & Roos, 1990; Pritchard & Dickie, 2003).

Applying ageing experiments is a valuable technique to compare the vigour of seed lots, both amongst and within species (Finch-Savage & Bassel, 2015). Accelerated or artificial ageing consists of exposing seeds to controlled high humidities and temperatures that lead to a loss of viability over relatively short periods of time (Roberts, 1973). After ageing seeds for different time intervals, seed germination tests are used to estimate the loss of viability. For this purpose, often the germination percentage is expressed in probit units, thus enabling viability loss to be plotted and analysed as a linear regression against ageing time (Finney & Tattersfield, 1952). The slope of that regression line is σ, i.e. the standard deviation of the distribution of seed deaths over time. The intercept, Ki, is the initial viability (germination) of the seed lot, as defined in Equation 7.1:

$$v = Ki - {}^{p}/_{\sigma} \qquad \text{Equation 7.1}$$

Where v is the viability, in probit units, after p days of seed storage. Ellis and Roberts (1980) have proposed several mathematical formulas to describe the quantifiable decrease in seed viability, named the viability equations. These have been used to predict seed longevity in a wide range of species with orthodox seeds using a broad range of artificial ageing conditions. The viability equation assumes two constants related to temperature and other two related to moisture that are species specific, the latter is usually expressed as log moisture content (Equation 7.2). The intercept, Ki, is expected to vary among species and seed lots. According to the viability equations, σ will be the same between seed lots of the same species. In reality, there is known to be some intra-specific variability in this parameter. Nonetheless, the effect of temperature and moisture on σ are expressed as follows:

$$\log \sigma = K_E - C_W \log MC - C_H T - C_Q T^2 \qquad \text{Equation 7.2}$$

where K_E and C_W are moisture constants and C_H and C_Q are temperature constants. The moisture content, MC, is measured for each seed lot and each ageing conditions and expressed on a wet weight basis (see Chapter 2, section 2.7.1). The temperature, T, is expressed in degrees Celsius (Roberts & Ellis, 1989).

Seed longevity can then be predicted from controlled storage conditions by combining the two previous equations (Ellis & Roberts, 1980):

$$v = Ki - {}^{p}/_{10^{K_E - C_W log MC - C_H T - C_Q T^2}} \qquad \text{Equation 7.3}$$

There are several studies that have explored the longevity of *Helianthus annuus* (sunflower) seeds, however in most cases using only one ageing condition or one constant temperature at several RHs or MCs (Ellis *et al.*, 1988, 1995; Kibinza *et al.*, 2006; Nagel & Börner, 2010; El-Maarouf-Bouteau *et al.*, 2011). This makes it difficult to compare any variability in longevity between seed lots or sunflower varieties as longevity results are reported in different time units, such as years or days. Other studies only report the time the seed takes to lose 50 % of viability (p50). To address these inconsistencies, in this chapter the effect of temperature and RH on seed ageing is explored using three temperatures in combination with up to four RHs.

The maternal environment is known to impact sunflower (and other species) seed viability (Fenner, 1991; Sanhewe *et al.*, 1996; Nasehzadeh & Ellis, 2017). Seed oil composition may vary with seed source environment (Unger & Thompson, 1982; Dornbos & Mullen, 1992; Wang & Frei, 2011) and that, potentially, will affect seed ageing as the equilibrium MC of the seeds will have changed. For example, seeds with greater lipid content and less starch will absorb less water as the lipid is hydrophobic (Harrington, 1972; Walters, 1998). This difference will be reflected in the seed oil content of the seed lots.

The temperature, photoperiod or plant diseases during seed development potentially influence the longevity of the mature seeds (Harrington, 1972; Schutte *et al.*, 2008; Nagel *et al.*, 2015). For example, Sanhewe and Ellis (1996) found that *Phasoleous vulgaris* plants growing at cooler temperatures produced seeds with greater longevity. In addition to different seed production environments, harvest time also affected Ki, and consequently seed longevity, in wheat and rice (Sanhewe *et al.*, 1996; Ellis, 2011). On the other hand, water stress during seed filling in soybean did not have a clear effect on seed germination and vigour (Dornbos &

Mullen, 1991). However, there are few studies which address how a reduction of water to the mother plant affects seed longevity and none for *Helianthus annuus*. Yet such studies are of fundamental importance when considering the future of agricultural production in Europe under a changing climate.

Physiological indications of the ageing process in seeds are delayed germination and an increased spread of germination over time, as well as poor conversion into normal seedlings (Ghassemi-Golezani *et al.*, 2010; Roqueiro *et al.*, 2010). In addition, aged seeds can have a reduced tolerance to sub-optimal environmental conditions or higher sensitivity to adverse conditions (Abdul-Baki & Anderson, 1972; Bradford *et al.*, 1993). The seed germination response to the environment can be described by population-based threshold modelling approaches such as thermal (temperature) and hydro (water potential) time (García-Huidobro *et al.*, 1982; Bradford, 1995). As reported in previous chapters, these modelling approaches describe the response of the whole seed population and as such provide a powerful tool to analyse the susceptibility of the seed germination process to environmental change. In this chapter, the thermal time (θ_T) and hydro time (θ_H) characteristics of the seeds will be calculated following ageing to observe possible changes on the germination models and their thresholds due to loss of viability. Argerich and Bradford (1989) found that θ_T could be used to describe ageing delayed germination and lowered germination rate in comparison to fresh and primed tomato seeds. Since it is expected that seed ageing will slow the germination of the seed lots, it is hypothesised that θ_T and θ_H will be longer while the seed lots lose viability in all ageing conditions. Moreover, the base temperature of a seed lot with high germination percentage can be lower than in a seed lot with low germination percentage of the same species (Abdul-Baki & Anderson, 1972). Thus, seed germination thresholds, base temperature (T_b) and base water potential (Ψ_b), may also change during seed ageing.

The importance of the conversion from seed germination into normal seedlings has been discussed in the previous chapters because seed germination tends to overestimate the development of seedling normality. This conversion is especially important for agricultural production and for

restoration programs when seeds are sourced from conventional seed bank conditions. Therefore, it is necessary to investigate the capacity of an aged seed to produce a normal seedling. Gay *et al.* (1991) identified the range of lethal temperatures, 40 to 45 °C, for seed germination and seedling emergence in non-aged seeds of *H. annuus*. There are few publications where the conversion into normal seedlings has been quantified on aged seeds (Bradford *et al.*, 1993). A parallel decrease of seedling normality and seed germination should be expected during ageing (Harrington, 1972). Indeed, it has been proposed that there are several approaches reflecting the different cellular capabilities as the ageing process progresses (Ellis & Roberts, 1984). For example, it is expected poor quality seeds will first lose the ability to produce normal seedlings, whilst still showing a positive reaction to the cell viability stain tetrazolium chloride (Abdul-Baki & Anderson, 1972; Ellis & Roberts, 1980), or as an accumulation of chromosome damage (Ellis & Roberts, 1984).

In this chapter data is presented on the ageing response of seed lots of *Helianthus annuus* genotype B harvested from plants grown under "normal" and "stopped irrigation" during the stage of seed filling (see Chapter 2 section 2.2.3). Studies within this research programme have shown that the maternal environment, had various effects on the seeds of *Helianthus* genotype B. For example, seed mass, base water potential and hydro time differed between both seed lots under the same germination conditions (see Chapter 6). Moreover, sufficient seeds were available (*c.*12,000) for the assessment of longevity, thermal and hydro characteristics of the seed lots. The objectives of this chapter are:

- To analyse the effect of the maternal environment (stopped and normal irrigation treatments) on seed longevity of *H. annuus* seeds.
- To estimate the viability constants for both seed lots at several seed ageing conditions at three constant temperatures in combination with four constant RHs.
- To explore whether seed germination thermal and hydro thresholds change during the ageing process as viability is lost.

- To compare seed vigour between the irrigation treatments, normal and stopped seed lots.

7.2 Materials and Methods

About 12,000 seeds of the crop genotype B of *Helianthus annuus* were used for the artificial ageing experiment. All seeds were harvested in 2015 in the south of Spain (Sevilla) by Limagrain seed company (France) and dispatched to Université Pierre et Marie Curie, UPMC (France). The seeds arrived at the Royal Botanic Gardens, Kew, Wakehurst Place in two batches. Batch 1 was received in 2015 and the seeds were kept at 15 °C and 15 % RH until use. Batch 2 was received nine months later, previously stored at 20 °C and 70 % RH at the UPMC. However once at Kew the seeds were dried at 15 °C and 15 % RH for at least 4 weeks. The seeds of both batches (from plants that had grown under "normal" and "stopped" irrigation conditions) were germinated at 20 °C to obtain their viability before starting the ageing experiment. When referring to the term seed lots this will include both Batch 1 and Batch 2 unless otherwise specified.

The seeds were equilibrated at 20 °C to obtain the predicted MC from the viability equations of Ellis and Roberts (1980) (see Chapter 2, Table 2.7). After equilibration the seeds were aged at three temperatures in combination with four RHs (see Chapter 2, Table 2.8).

To test seed viability at each time interval, 75 seeds were germinated at 20 °C. Moreover, 375 seeds were taken at t_1, t_2 and t_4 time intervals to perform a thermal time (θ_T) analysis from seeds germinating at 10 °C, 20 °C and 25 °C ±2 °C and hydro time (θ_H) analysis from seeds germinating at 0, -0.3 MPa and -0.5 MPa at 20 °C (see Chapter 2 section 2.7 for more details).

Germination as defined by radicle emergence was used to assess seed viability, but the proportion of normal seedlings (PNS, %) were also recorded (Chapter 2, section 2.7.3).

The statistical analyses performed to compare the seed lots and the intervals of ageing were analysis of variance (ANOVA), Tukey test and t-tests. Percentages were arcsine transformed to perform comparative

analysis (ANOVA and t-test). GenStat 12.1 software (VSN International Ltd, 2009) was used for probit analysis on the data obtained from thermal and hydro time models as described in Chapter 2.

7.3 Results

7.3.1 Effect of equilibration at different RHs at 20 ℃ on seed viability

Equilibrating seeds to different RHs at 20 ℃ had no effect on seed viability (based on seed germination percentage) (Table 7.1) nor germination rate (Figure 7.1) for either seed batches. Although Batch 2 was received 9 months later than Batch 1 the viability and germination rate did not significantly differ between them.

Table 7.1 Comparison of seed germination on fresh seeds and equilibrated seeds at 20 ºC of *Helianthus annuus* genotype B. Seed germination (G %) was calculated on seeds from two plant production treatments (normal and stopped irrigation). Seeds were assessed either prior to (fresh) or after equilibration at 20 ℃ to different RHs for the ageing experiments at different temperatures (see Chapter 2, Table 2.8). The results of ANOVA test are in Appendix Table A7.2.

Condition (℃ - RH)	Normal irrigation G (%)	Stopped irrigation G (%)
Batch 1		
Fresh	86.6 ± 9.0	98.0 ± 3.5
40-30	86.7 ± 5.0	97.3 ± 1.9
30-45	90.7 ± 1.9	90.7 ± 3.8
20-75	84.3 ± 3.0	92.1 ± 3.3
20-60	90.7 ± 1.9	94.7 ± 1.9
Batch 2		
Fresh	84.48 ± 7.9	94.6 ± 7.8
40-75	70.7 ± 1.9	97.3 ± 3.8
40-60	78.7 ± 8.2	85.3 ± 3.8
40-45	86.7 ± 6.8	96.0 ± 1.9
30-75	80.0 ± 6.5	86.7 ± 10.0
30-60	88.0 ± 0.0	88.0 ± 3.3

7.3.2 Moisture content during ageing experiment

To monitor seed MC during ageing, MC was measured at the beginning of the ageing experiment (t_1, after seed equilibration at 20 ℃, see Chapter

2, Table 2.8) and at the end (t_5, last time interval studied). A significant decrease in MC was observed at t_5 compared with t_1 (Table 7.2) in eight out of 18 ageing conditions (i.e. 44%), averaging a 0.6% MC difference. These differences were observed in both normal and stopped irrigation seed lots. Furthermore, seeds of the stopped irrigation seed lot had significantly lower MC than those of the normal irrigation seed lot at the beginning of the ageing experiment (t_1) in four ageing conditions (*Appendix* Table A7.3).

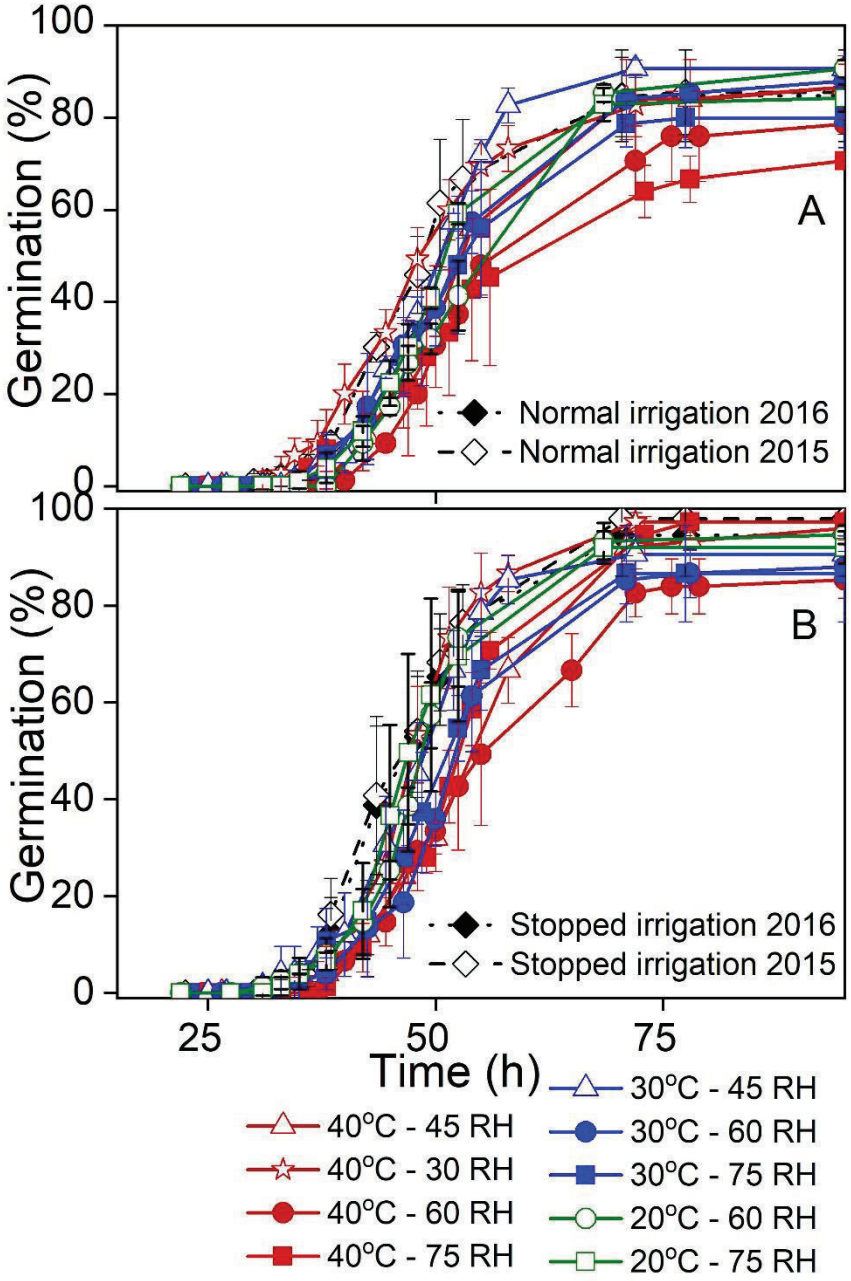


Figure 7.1 Cumulative germination of seeds of two seed lots of *H. annuus* genotype B, normal irrigation (A) and stopped irrigation (B). Data is shown for fresh seeds and after equilibration to different RHs at 20 °C (see Table 7.1). In black are the germination percentages of the fresh seeds of Batch 1 (seeds received on 2015, open diamonds) and the Batch 2 (seeds kept for 9 months at 70 % RH, solid diamonds). Where the error bars are not visible, the SD is smaller than the size symbols.

Table 7.2 Comparison of the seed moisture content (MC %) between the initial time interval (t_1) and last time interval (t_5) of the ageing experiment. The MC values, on wet weight basis, are the means (± SD) of 10 seeds for each ageing condition for the seed lots from plants of *H. annuus* subjected to two water treatments (normal and stopped irrigation). The asterisks (*) denote significant differences ($P < 0.05$) between t_1 and t_5.

		MC (%)	
		After eRH t_1	**End of ageing t_5**
40°C – 75 % RH	Normal	8.179 ± 0.18	7.701 ± 0.46
	Stopped	7.620 ± 0.14	7.425 ± 0.40
40°C – 60 % RH	Normal	6.249 ± 0.19	5.993 ± 0.35
	Stopped	5.760 ± 0.08	5.434 ± 0.34
40°C – 45 % RH	Normal	5.188 ± 0.11	4.702 ± 0.25*
	Stopped	4.776 ± 0.09	4.445 ± 0.22*
40°C – 30 % RH	Normal	4.646 ± 0.39	3.980 ± 0.20*
	Stopped	4.368 ± 0.30	3.813 ± 0.16*
30°C – 75 % RH	Normal	8.564 ± 0.14	8.171 ± 0.81
	Stopped	7.620 ± 0.14	7.192 ± 0.36
30°C – 60 % RH	Normal	7.125 ± 0.15	6.233 ± 0.27*
	Stopped	6.677 ± 0.24	5.850 ± 0.30*
30°C – 45 % RH	Normal	5.802 ± 0.32	4.984 ± 0.39*
	Stopped	5.431 ± 0.44	4.975 ± 0.24*
20°C – 75 % RH	Normal	8.628 ± 0.18	8.357 ± 0.51
	Stopped	8.335 ± 0.49	7.933 ± 0.71
20°C – 60 % RH	Normal	7.129 ± 0.59	6.771 ± 0.52
	Stopped	7.078 ± 0.41	6.480 ± 0.51

7.3.3 Seed longevity

The predictions of seed viability from the viability equations (Equation 7.3), were not accurate for most of the ageing conditions (*Appendix* Table A7.1). Seeds of the stopped irrigation treatment had higher initial germination than those of the normal irrigation treatment (Table 7.1). Moreover, the two seed lots (normal and stopped irrigation) seem to age at different rates (Figure 7.2 and Figure 7.3). For example, seeds of the normal irrigation treatment reached the 50th percentile of germination in less time than those of stopped irrigation treatment. Thus, it was difficult to achieve a similar percentage of germination at the same time intervals for all ageing conditions.

Total seed germination percentage of each ageing condition was transformed into probit units using tabular data from Finney and Tattersfield (1952). The 50th percentile of germination (in this instance to represent half viability) is represented by probit 5. Seeds of both treatments of *H. annuus* (stopped and normal irrigation) were affected by the ageing conditions (Figure 7.2 and 7.3). Furthermore, the slopes of the regression lines of both seed lots were similar in most cases (*Appendix* Table A7.4). Seeds took longer time to lose viability when aged at low temperatures and RHs. When comparing different RH at the same temperature, seeds stored at the lowest humidity needed more time to lose 50 % of viability (e.g. 30 % RH at 40 ℃, Figure 7.2A). On the other hand, when comparing different temperatures at constant RH, seeds stored at 20 ℃, the lowest temperature studied, lived longer (Figure 7.3).

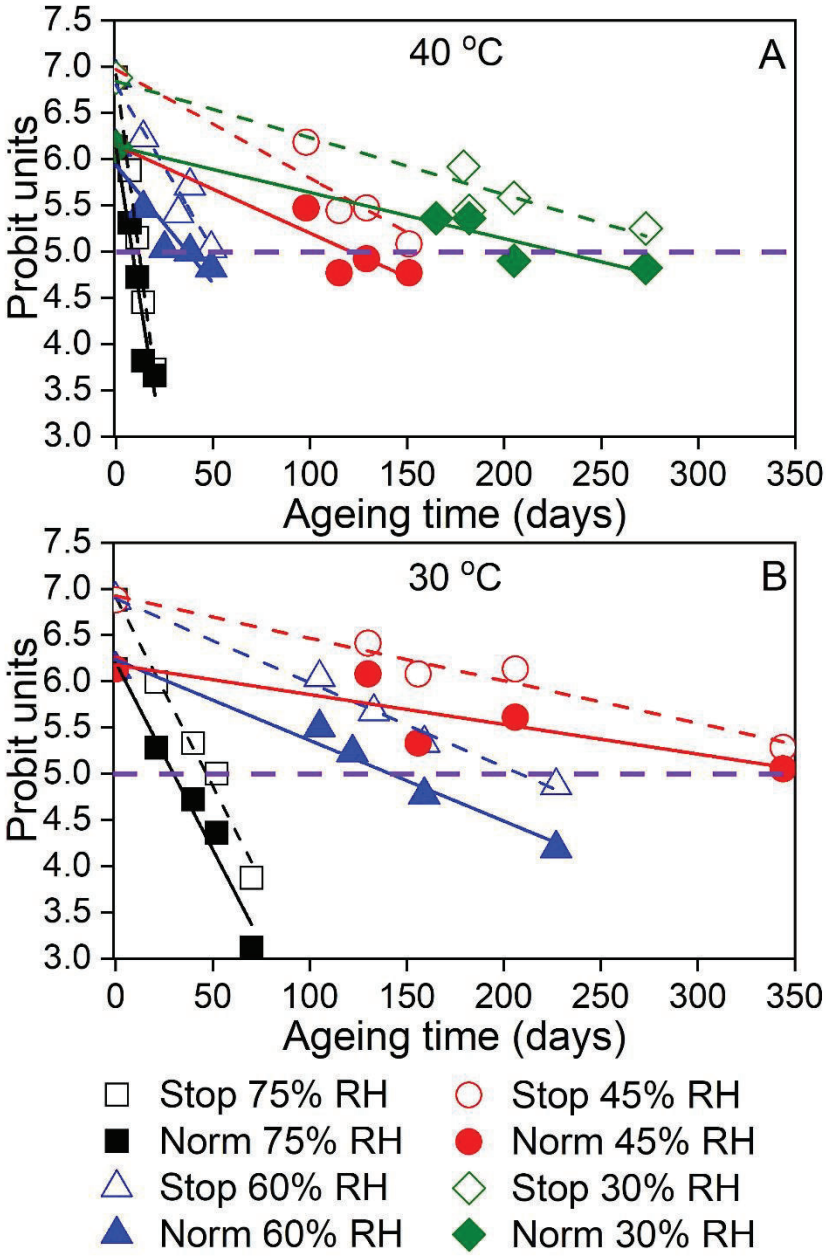


Figure 7.2 Linear fit of the final germination transformed into probit units for each ageing condition on *H. annuus* seeds at 40 °C (A) and 30 °C (B) and four relative humidities (RH) at five time intervals (Chapter 2, Table 2.8). Artificial ageing was performed in seeds of *H. annuus* genotype B in two treatments, stopped irrigation (Stop, dash lines) and normal irrigation (Norm, solid lines). The 50th percentile of viability (5 probit units, p50) is highlighted with a purple dash line. The equations of the regression lines are displayed in the *Appendix* Table A7.4.

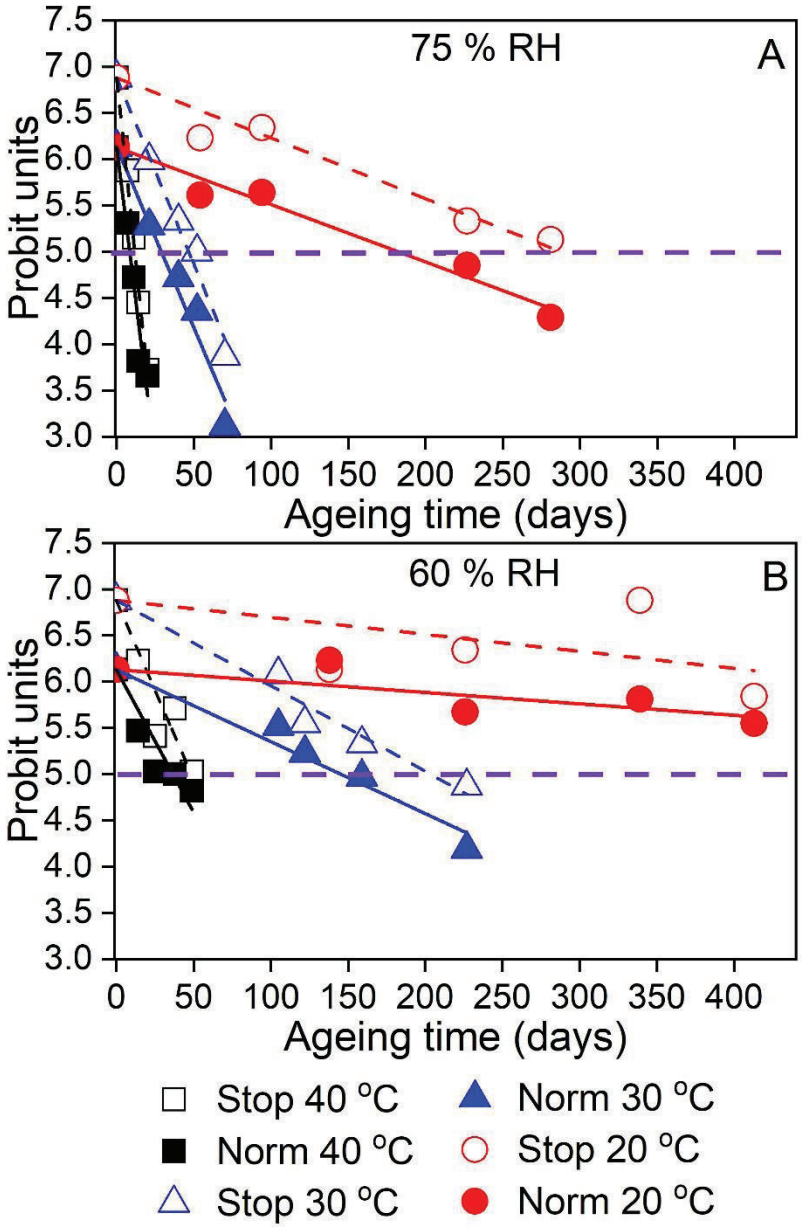


Figure 7.3 Linear fit of the final germination transformed into probit units for each ageing condition on *H. annuus* seeds at 75 % relative humidity (RH, A) and 60 % RH (B) at five time intervals (see Chapter 2, Table 2.8). Artificial ageing was performed in seeds of *H. annuus* genotype B in two treatments, stopped irrigation (Stop, dash lines) and normal irrigation (Norm, solid lines). The 50th percentile of viability (5 probit units, p50) is highlighted with a purple dash line. The equations of the regression lines are displayed in the *Appendix* Table A7.4.

The time that a seed lot took to lose 50 % of viability (p50) was estimated from the longevity curves based on the final germination for each ageing condition. Seeds of the stopped irrigation treatment had longer p50s compared with those of the normal irrigation treatment under the

same ageing conditions (Figure 7.4 and 7.5). The regression lines showed different slopes in the two seed lots at the same temperature (*Appendix* Table A7.5). Nonetheless, at the same RH the slopes of the regression lines of normal and stopped irrigation were similar. Therefore, with a decrease of 5 °C the p50 values reduced at the same rate in both seed lots (Figure 7.5). However, a decrease of *c.* 10 % RH results in a greater increase of p50 in the stopped irrigation than in normal irrigation seed lot (Figure 7.4). In other words, the benefit of decreased RH to low values on seed longevity appeared to be greater in the seeds harvested from plants subjected to "stopped irrigation".

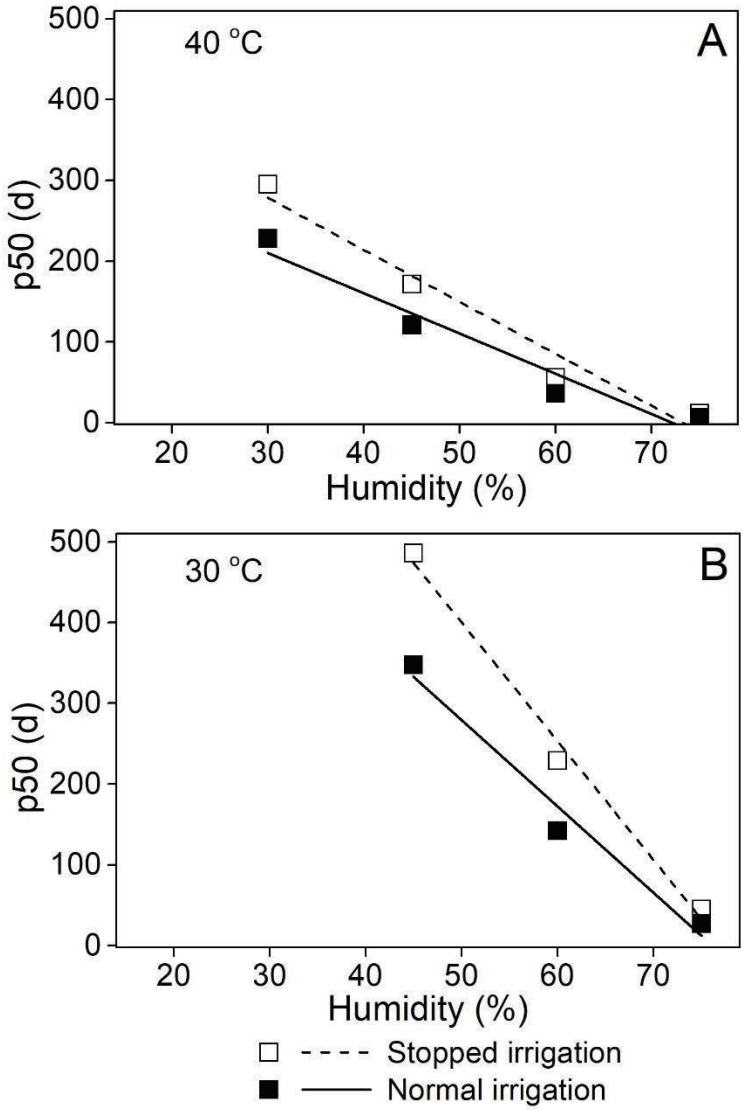


Figure 7.4 Linear regression between the days of ageing to reach 50 % viability (p50) and relative humidity (%) at the same temperature, 40 °C (A) and 30 °C (B). The responses of seeds of two treatments (stopped and normal irrigation) of *H. annuus* genotype B are shown. The equations of the regression lines are displayed in the *Appendix* Table A7.5.

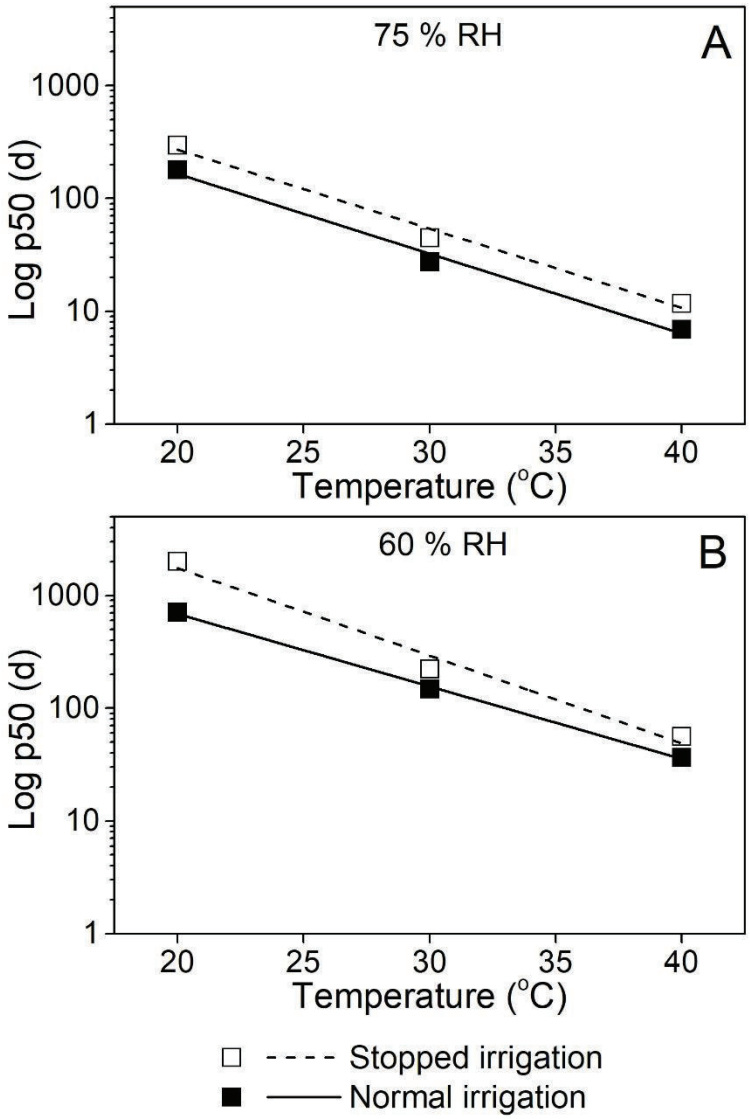


Figure 7.5 Linear regression between the days of ageing to reach 50 % viability (p50) on a logarithmic scale and the temperature (°C) at the same relative humidities (RH) of 75 % (A) and 60 % (B). The responses of seeds of two treatments (stopped and normal irrigation) of *H. annuus* genotype B are shown. The equations of the regression lines are displayed in the *Appendix* Table A7.5

The p50 calculated from the previous regression lines at the same temperatures (20, 30 and 40°C) were plotted on a logarithmic scale against the logarithm of mean MC between t_1 and t_5 (Figure 7.6). A common regression line for both seed lots at each temperature was found. Thus, the effect of reducing MC on increasing p50 under three storage temperatures (20, 30 and 40°C) is the same for both seed lots.

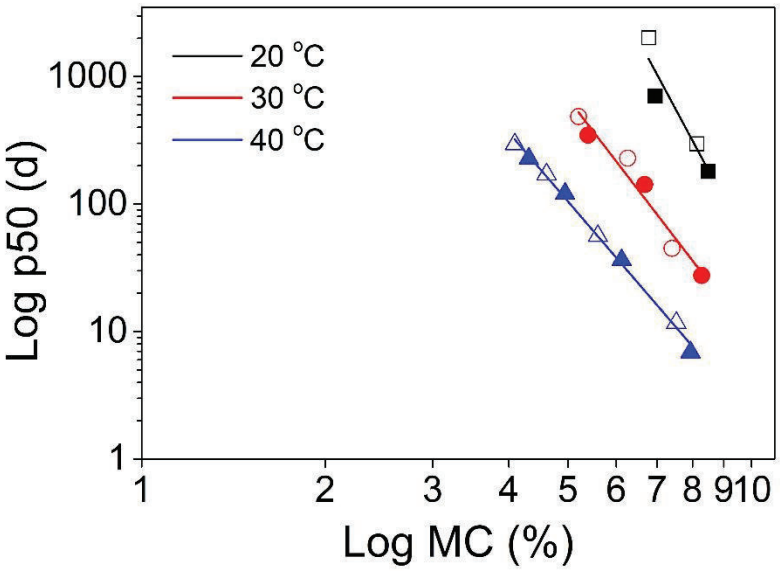


Figure 7.6 Representation of longevity by p50 (days for seed lots to lose 50 % of the viability) in a logarithmic scale. The effects of the mean moisture content (MC) in a log scale and the temperature of storage on seed longevity of both seed lots of *H. annuus,* stopped (open symbols) and normal irrigation (solid symbols) are shown. The equations of the linear regressions are for 20°C, y = -8.9237x + 10.55578 (r = -0.95, P > 0.05); 30°C, y = -6.2294x + 7.18163 (r = -0.98, P < 0.001); and 40°C, y = -5.56975x + 5.91361 (r = -0.99, P < 0.001). The degrees of freedom (DF) are the number of data points minus 2 for each temperature.

Ki (intercept) and σ (slope) were obtained from the longevity regression lines of Figure 7.2 and 7.3, to calculate the provisional viability constants (i.e., K_E and C_W as moisture constants and C_H and C_Q as temperature constants) for each seed lot using the Equation 7.2 (Table 7.3).

Table 7.3 Provisional seed viability constants for *Helianthus annuus* calculated for each seed lot from plants subjected to normal and stopped irrigation. K_E and C_W are moisture constants and C_H and C_Q are temperature constants (± SD). ⸸The constants of sunflower as presented in the Seed Information Database of Kew from Ellis *et al.* (1988). They were the first values used to predict the time of storage described in the *Appendix* Table A7.1.

Viability constants	Normal irrigation	Stopped irrigation	Sunflower constants⸸
K_E	6.37 ±0.11	5.51 ±0.16	6.74
C_W	4.15 ±0.12	3.52 ±0.19	4.16
C_H	0.0495 ±0.0035	0.0249 ±0.0054	0.0329
C_Q	0.000114 ±0.00012	0.000451 ±0.00018	0.000478

7.3.4 Thermal time and hydro time during artificial ageing

Thermal time (θ_T) and hydro time (θ_H) were calculated from the germinated seeds (radicle emergence) of three time intervals, t_1, t_2 and t_4 (see Chapter 2, Table 2.8). In general, the germination rate was slowed, and germination was delayed as the artificial ageing experiment progressed (*Appendix* Figure A7.1). This was reflected by larger values of θ_T for seeds under ageing conditions for the longest time (t_4), in contrast to seeds from the first two time intervals (t_1 and t_2) or fresh seeds with higher viability (*Appendix* Table A7.6). However, there were some exceptions at 20 ºC - 60 % RH, 30 ºC - 45 % RH, 30 ºC - 60 % RH, 40 ºC - 30 % RH and 40 ºC - 45 % RH. These were due to large variance of θ_T values for each seed lot and also to small or non-significant ($P > 0.05$) changes in viability during intervals, especially for seeds of the stopped irrigation treatment (i.e. 20 ºC - 60 % RH, *Appendix* Table A7.6).

The seed germination thresholds (T_b and Ψ_b) did change during ageing, in particular T_b was higher when the viability was lower in all ageing conditions with the exception of 40 ºC - 60 % RH (both seed lots) and 40 ºC -75 % RH, 40 ºC - 30 % RH, 30 ºC -75 % RH and 20 ºC -75 % RH (only seeds of the normal irrigation treatment). Nonetheless, the viability

(probit values) was negatively correlated with T_b (*Appendix* Figure A7.2). The values of Ψ_b tended to decrease at longer time intervals with the loss of viability. However, due the large variability of the seed lots some of the differences between time intervals were not significant (*Appendix* Table A7.6).

A significant negative linear correlation was found when viability (probit units) was plotted against θ_T (Figure 7.7A) and θ_H (Figure 7.7B) of each ageing condition and time interval. This means that there is a systematic lengthening of θ_T and θ_H of seeds as ageing decreased the population viability for *c.* 98% to 50%.

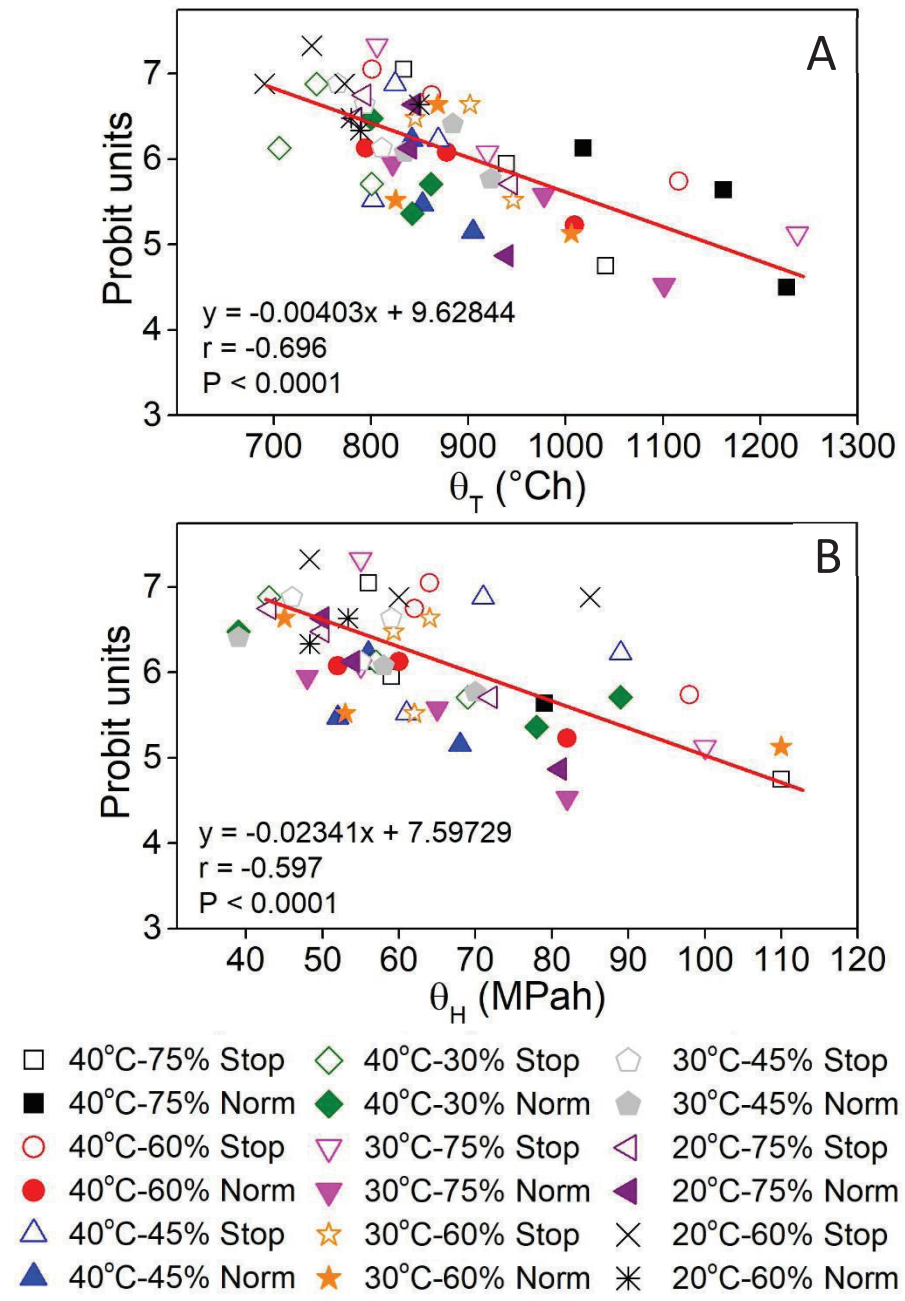


Figure 7.7 Correlation between the viability in probit units and thermal and hydro time for each ageing condition. The viability of the time intervals t_1, t_2 and t_4 was transformed into probit units and plotted against A) thermal time (θ_T) and B) hydro time (θ_H) combining both stopped irrigation (Stop, open symbols) and normal irrigation (Norm, solid symbols) seed lots.

7.3.5 Conversion of germinated seeds into normal seedlings

As observed in Chapter 6, the proportion of normal seedlings was lower at 25 ℃ than at 10 or 20 ℃ for each ageing condition. In this chapter the effect of ageing on seedling normality is explored. At some ageing conditions, the proportion of normal seedlings decreased with the loss of viability (*Appendix* Table A7.7). For example, seeds aged at 40 ℃ - 75 % RH of the normal irrigation treatment germinated up to 70.7 % at t_1; 92.7% of these germinated seeds developed into normal seedlings at 10℃. However, in t_4 (after 14 days of ageing), 12.7% of the seeds germinated and only 53% of them developed into normal seedlings. Percentage normal seedling in proportion of the germinated seeds tend to decrease while the viability is lost. Those decreases were not always significant due to a large standard deviation.

7.4 Discussion

According to gene bank standards for drying and storage, the preferred conditions for the long-term storage of seeds are -18 ℃ following equilibration to 10 – 25 % relative humidity, RH (FAO, 2014). Such conditions generally extend seed longevity to many years. Consequently, to study the potential effects of the maternal environment in which the seeds matured on seed longevity, it was critical to accelerate the ageing rate of seeds. To do this, seed moisture content (MC) and temperature were increased and the seed longevity was determined over a range of abiotic conditions and times (up to 9 ageing conditions).

7.4.1 Effect of Ki and MC

Quantifying the impact of the abiotic environment on seed longevity is an important approach for comparing the developing perspective on how long the seeds might survive in storage, including gene banks (Pritchard & Dickie, 2003). Because seed longevity is sensitive to both humidity and temperature, controlling them during ageing tests is a concern. Firstly, to start an ageing experiment the seeds need to be equilibrated to a specific MC, generally by placing the seeds above salt solutions at different RHs (Roberts & Ellis, 1989) and at constant temperature. Secondly, there is the

possibility that during the ageing experiment the seed MC might change due to the deterioration of the seed (Vertucci & Roos, 1993; Walters, 1998). In this chapter, the effect of equilibrating the seeds at specific MC at 20 ℃ on the initial viability of the seed lots was proved null. There was no effect at any ageing conditions studied here in either normal or stopped irrigation *H. annuus* seed lots.

Sinniah *et al.* (1998), found that limiting the irrigation to *Brassica* plants after flowering stage, increased their "potential longevity". Similarly, according to the regression lines of Figure 7.2 and 7.3, seeds of the stopped irrigation treatment appeared to have the capacity to live longer than seeds of the normal irrigation treatment. However, on closer inspection it seems that the main effect is that seeds of the normal irrigation started with a lower viability (Ki). In fact, the ageing experiment had the same effect on both seed lots since the regression lines for viability loss are parallel. However, the differences on Ki were mitigated when the seed MCs were compared (Figure 7.6). In other words, when the seed lots are equilibrated at the same MC, they will age at the same rate, independently of the Ki. This is probably because in this specific case, the differences of Ki between both seed lots are less than 15 %.

Seeds of the stopped irrigation treatment had significant higher oil content than those of the normal irrigation treatment (41.9 and 38.7 % respectively) as described in Chapter 3. Based on the known dependency of water sorption properties of seeds on chemical composition, and in agreement with Vertucci and Roos (1990) and Pritchard and Dickie (2003), the MC of seeds with the higher oil content (i.e., stopped irrigation seed lot) was generally lower than those with a lower oil content (i.e., normal irrigation seed lot). On the other hand, the differences in MC between both seed lots were only significant for the ageing treatments where seeds of Batch 2 were used (*Appendix* Table A7.3). Priestley (1986) and Lins *et al.* (2014) observed a decrease of seed oil content with the loss of viability due to lipid peroxidation and possible fungal infection that degraded the fatty acids reducing the total oil content (Robertson *et al.*, 1984; Bhattacharya & Raha, 2002). Fungal infection has been identified in oilseeds resulting from high storage humidity (Robertson *et al.*, 1984;

Roberts *et al.*, 1986; Bhattacharya & Raha, 2002; Kakde *et al.*, 2012). Fungal growth has been reported at water activities superior to 0.6 (Stevenson *et al.*, 2015). This suggests that the storage of seeds under relatively wet conditions of 70% RH for 9 months, as seeds of Batch 2 were stored before the start of the experiment, may increase the differences between seed lots, particularly seed oil content, and that it may be reflected in the MC of the seeds. However, oil content and differences in MC between the two seed lots is not thought to have a large impact on any differences in seed longevity observed.

For the prediction of seed longevity to be accurate over time, it is important that seed MC is maintained during ageing. Any changes in MC during storage will impact potentially on the ageing rate. Whilst it is assumed the MC does not change (Roberts & Ellis, 1989), increases in seed MC at lower storage temperatures have been reported (Vertucci & Leopold, 1987; Ellis & Hong, 2006). Presumably because at a given RH, less water is adsorbed at higher temperatures (Cromarty *et al.*, 1982; Vertucci & Leopold, 1987). In this study, seed MC was measured at the beginning (t_1) and at the end (t_5) of the ageing experiments for each condition in both *H. annuus* seed lots (normal and stopped irrigation). The equilibration temperature was 20 °C and the ageing experiments were at 20, 30 and 40 °C. Differences on the MC could be expected at all ageing conditions of 40 °C and 30 °C because the equilibrium temperature was not the same. However, this was not the case (Table 7.2), with seeds maintaining their pre-storage MC even when the storage temperature was increased by 20 °C (i.e. up to 40 °C).

In summary, the seed MC was controlled when possible with differences during storage of < 1 % MC. These changes seem not to have had a significant effect on the ageing parameters. The lifespan of seeds from stopped irrigation seed lot is longer than those harvested from the normal irrigation seed lot, mainly due to having a higher Ki and slightly lower MC as a result of different oil contents. Consequently, I explored how the factors of MC, oil content and storage temperature impacted the ageing kinetics of the seeds through the determination of the viability equation constants.

7.4.2 Viability equations

The seed viability equations developed by Ellis and Roberts (1980) have been used to model and predict seed longevity, within certain moisture and temperature conditions. Some discrepancies may exist when dealing with conditions beyond those used to develop the viability constants. An example is the effects of ultra-drying (Vertucci & Roos, 1990; Mira *et al.*, 2015). Nonetheless, it is possible to gain greater understanding of seed longevity from taking a viability equation approach (Pritchard & Dickie, 2003). In this instance, the interest was to see if the relative effect of MC on seed ageing varied in seed lots produced on plants growing under different agronomic conditions.

From the predicted longevity regression lines for a specific temperature and RH it is possible to estimate the longevity of these seed lots at constant and controlled storage conditions. The predictions of p50 on *H. annuus* seeds aged at constant temperature suggest 1 % decrease in MC, increases seed longevity by 2.6 times. For example, seeds with 7 % MC at 40 ºC lost 50 % of viability after 17 days; whereas seeds with 1 % MC lower needed 44 days to lose 50 % of viability. This relationship is in broad agreement with Harrington (1972) who suggested that seed life span doubles for each 1 % fall in MC. This prediction is known to hold reasonably well within the range of 2 % to 16 % MC (Ellis *et al.*, 1989; Roberts & Ellis, 1989), and the range of MC used in this chapter is within that range (between *c.* 4% to 9 %).

Some studies suggest that the temperature parameters are constant within species (Ellis & Roberts, 1980; Dickie *et al.*, 1990). Sunflower viability constants were calculated by Ellis *et al.* (1988) at different MC and one temperature. Thus, they assumed the temperature parameters (C_H and C_Q) to be the same as for other species. In contrast, in this chapter all the viability constants were calculated for each seed lot using the Equation 7.2 (Table 7.3). C_W is a moisture constant that varies with seed oil content and the values range between 2 and 6 (Ellis *et al.*, 1990). Seed lots with higher oil content as the stopped irrigation seed lot, have lower C_W value (Table 7.3). From that is possible to deduce that the sunflower seed lot that Ellis *et al.* (1988) used had a similar seed oil content than the normal

irrigation seed lot (C_W = 4.16 and 4.15 respectively) but lower than the stopped irrigation seed lot. The other moisture constant K_E, indicates the susceptibility to lose viability. Thus, it is a constant that will vary between seed lots. In this case, the stopped irrigation seed lot shows a lower K_E value than the normal irrigation seed lot, indicating higher seed longevity when stored under the same conditions.

However, when these provisional constants were applied to the Equation 7.3, the prediction of the storage period was not accurate to the current ageing experiment for any seed lot. The lack of accuracy could be due to a narrow range of temperatures and MC. More ageing conditions will be needed to have an accurate result of the viability constants. On the other hand, the predictions of sigma (σ, days to lose one probit of viability) were accurate. The seeds from the stopped irrigation seed lot needed more time (12 days more) to lose one probit value than normal irrigation seed lot when aged at 20 ℃ and 75% RH.

In summary, it seems that there are subtle differences in the seed viability responses of the seeds from plants grown under different conditions and that these are manifest as changes in the viability constants. Similarly, other studies have shown differences in all four viability constants within the same species amongst different cultivars or different seed maturation stages or harvesting times (Zanakis *et al.*, 1994; Hay *et al.*, 1997; Tang *et al.*, 1999). This suggests some limitations to the assumption that published viability constants can accurately predict seed longevity in all seed lots of a species.

7.4.3 Seed germination thresholds (thermal and hydro) varied during seed ageing

One consequence of seeds losing viability is that the germination rate of those that can still germinate is lower (Ellis & Roberts, 1984). For example, the mean time to germinate becomes longer (Ellis & Roberts, 1980; Argerich & Bradford, 1989). There are at least two thermal parameters of the germination response that could contribute to slower germination in aged seeds. Firstly, the threshold temperature for germination could be increased with ageing whilst the thermal time (θ_T)

for germination remained the same. Secondly, the thresholds could remain whilst the thermal time lengthened. A combination of both might also occur. Similarly, the water relations of seed germination could change ageing time.

The loss of seed viability contributed to spread seed germination over time. In agreement with Abdul-Baki and Anderson (1972) and Bradford *et al.* (1993), the T_b threshold seems to be reduced with loss of viability. The T_b of t_4 was higher than in t_1 in both seed lots and θ_T and hydro time (θ_H) increased with the loss of viability (*Appendix* Table A7.6). However, those differences were not always significant due to the high variability of the seed lots during seed ageing. Seed viability did not decrease after more than 300 days as observed at 20 ºC - 60 % RH (t_4) and the θ_T was statistically the same between t_1, t_2 and t_4 in both seed lots (*Appendix* Table A7.6). However, T_b and θ_H values differed among the three ageing intervals (t_1, t_2 and t_4). As, T_b and θ_H are sensitive to seed ageing, even if it has not been manifested through loss of viability, these seed germination parameters could be used as indicators of seed ageing before the viability declines in *H. annuus* seed lots.

A possible effect of the models is observed. When calculating θ_T and θ_H parameters in the repeated probit analysis, θ_H and T_b are the "constant" parameters for all percentiles of the population (Bradford, 1995). In contrast, θ_T (inverse of the slope) varies at different percentiles (among seed population, *Appendix* Figure A7.3). Thus, θ_T is more variable within a population, or in this case within each ageing condition (period length of seed ageing) and covers a wider range of values than T_b or θ_H (*Appendix* Figure A7.3). Therefore, it is expected that the variance of θ_T between seed ageing conditions will be large. As a consequence of large variance among the replicates, differences in θ_T were not significant at some ageing conditions (e.g. 40 ºC – 75% and 60% RH in normal irrigation seed lot and 20 ºC - 75 % RH, *Appendix* Table A7.6).

The probit values of germination percentages (i.e. viability) for each ageing interval and condition were negatively correlated with θ_T, θ_H and T_b (Figure 7.7 and *Appendix* Figure A7.2). Both seed lots had similar slopes, and it was possible to plot one general regression line describing

the relationship between viability (i.e. total germination as radicle emergence in probit units) and θ_T, θ_H or T_b. These regressions demonstrate that the viability of the seed population is a marker for the subsequent speed of germination under different temperature and water potential conditions. Moreover, seed lots with lower viability have altered thermal and hydro time parameters and performance. Previous studies have shown a relationship between the loss of viability and an increase in mean time to germination, MTG (Ellis & Roberts, 1984; Argerich & Bradford, 1989). On the contrary to θ_T and θ_H, MTG is not related to the thresholds, T_b nor Ψ_b. Thus, as Soltani *et al.* (2016) described MTG is not the right measurement of vigour when comparing different seed lots or treatments. For the calculation of θ_T, θ_H and their thresholds for each ageing condition, 50th percentile (t50) is used as reported in previous chapters. The results presented in this chapter supports the use of germination rate as a predictor of vigour and viability of a seed lot (Argerich & Bradford, 1989) more specifically using θ_T, when germinating seeds at several temperatures, and θ_H, when germinating the seeds at several water potentials. The predictive value of these changes remains to be seen. Nonetheless, it is known that the loss of one probit viability in seeds of *H. annuus* genotype B, is accompanied by an increase in θ_T by 248 °Ch and in θ_H by 43 MPah (Figure 7.7).

Seed vigour has been widely described as a complex trait that will include several properties such as homogeneous and fast germination and development under wide range of environments (Pollock & Roos, 1972; Rajjou *et al.*, 2012; Finch-Savage & Bassel, 2015; ISTA, 2017) including stressful conditions such as seed ageing. In this chapter it is shown that θ_T and θ_H during seed ageing are comprehensive parameters that could be used as descriptors of seed vigour. These germination parameters help to describe the seed viability, the speed of germination under constant and sub-optimal temperatures and water potentials and their thresholds.

7.4.4 Seed ageing affects normal seedling growth

The effect of seed ageing can be characterised by several approaches (Ellis & Roberts, 1984). For example, as the loss of the ability of cells of

the embryo to stain with tetrazolium chloride or as the lack of capacity of a seed to growth (Ellis & Roberts, 1984). In agreement with the findings in this chapter, an overestimation of viability was found when percentage radicle emergence was compared to percentage normal seedling (Popova *et al.*, 2013; Ballesteros & Pence, 2017). Thus, a loss of the viability of the seed lots (as radicle emergence) is often followed by a decrease in normal seedling development (Abdul-Baki & Anderson, 1972). The appearance or activation of some fungi during *H. annuus* seed storage has been observed (Lins *et al.*, 2014). Both seed lots of *H. annuus* genotype B were previously infected by *Rhizopus* sp. fungi at the same level (54 - 57 %, see Chapter 3 and Chapter 6). However other kind of fungi such as *Aspergillus* sp. and *Penicillium* sp. could develop during artificial storage (Lins *et al.*, 2014) and have greater effects on normal seedlings in seed lots with lower vigour and protection (e.g. antioxidant capacity).

In this chapter, the proportion of germinated seeds that converted into normal seedlings was variable; decreasing in some cases, constant in others (*Appendix* Table A7.7). The variability of the seed lots was also influential when seedling normality was compared, reflected in the large standard deviations of the normal seedlings. On the other hand, when the interval of confidence of the statistical analysis (Tukey test) was 90 % (i.e. $P < 0.1$), normal seedlings of t_4 were significantly lower than those of t_1 in the majority of non-exceptional cases. Usually seedling normality at day 0 (time interval t_1) was as high as expected from Chapter 6. However, there were some exceptions, especially when the seeds were germinated at 25 ºC after 40 ºC – 45 % RH and 40 ºC – 60 % RH conditions to both seed lots. This was unexpected and the temperature records of the incubator at 25 ºC did not suggest any anomalies regarding temperatures nor light during germination experiment. Other factors such as distilled water, germination paper and Petri dishes were the same for all the germination conditions used on the time interval t_1 and the seedling normality was as expected. Thus, a deficient sample of seeds could be the reason to the poor conversion of germinated seeds into normal seedlings in that case.

Similarly, Salicaceae seeds after various conditions of ageing, including drying and exposure to ultra-low temperature, produced fewer normal

seedlings than emerging radicles (Popova *et al.*, 2013). Additionally, Bradford *et al.* (1993) found a significant decrease in normal seedlings in comparison to germinated seeds at longer periods of ageing in lettuce seeds. These findings are in agreement with the observations in this chapter where the conversion from germinated seeds into normal seedlings tends to be lower with loss of viability (*Appendix* Table A7.7). This means that there can be attrition (seed loss) as the germination process progresses. For this reason, seedling normality as well as seed germination (i.e., radicle emergence) is the preferred means of assessing seed vigour and seedling survival in the seed trade (ISTA, 2017). Thus, estimates of normal seedlings post-storage is extremely important for plant regeneration and restoration programmes.

8 CHAPTER 8: GENERAL DISCUSSION

8.1 Rationale of the project and main findings

Seeds are one of the main plant dispersal units and therefore essential to ensure the persistence of a species. Seed functional traits such as seed germination and longevity are of great importance to food security and agriculture. Nowadays the interest in preserving wild species, especially crop wild relatives (CWR), is increasing due to the recognition that they possess useful functional traits for crop improvement. However, fluctuations in the environment produced by climate change are threatening the habitat of CWRs (Jarvis *et al.*, 2008; Aguirre-Gutiérrez *et al.*, 2017) and it is likely these new environmental conditions might not be suitable for CWR survival. Additionally, extreme environmental events might eradicate some individuals reducing the populations of CWRs and the genetic diversity. Environmental changes are also threatening food security through yield loss (Lobell & Field, 2007; FAO, 2010; IPCC, 2013). The intergovernmental panel on climate change (IPCC) described climate scenarios for the next 50-80 years. Among their predictions it is stated: precipitation will be scarce in the Mediterranean and Southern Europe, while in Northern and Central Europe and the East of North America precipitation will increase. Furthermore, increments of *c.* 3 °C are expected in North America, Europe and the Mediterranean region (IPCC, 2013).

Despite these climate predictions being widely publicised, the environmental impacts on seed functional traits have not been fully explored or understood. What is known is that seed dormancy and germination are key traits to assess when considering the direct or indirect effects of environmental change (Walck *et al.*, 2011). To address the limitation in understanding germination performance in relation to environmental change the intention of this thesis is to expand the knowledge on how the environment (temperature and water) may impact on seed functional traits in CWRs of three important crops: *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus*. This was achieved in two main ways. Firstly, by characterising seed lots of a range of CWRs

from different environments and by correlating historical climate data from the environment of the seed collection site with the seed functional traits determined (Chapters 3, 4, 5 and 6). Secondly, three crop species were grown under not-limiting irrigation and under reduced watering. In addition to the maternal environment, several "germination environments" that differed in temperatures and water potentials were used to characterise the seed functional traits.

The main findings of this thesis are as follows:

- Crops seeds had higher mass and larger seeds (embryo length and endosperm length) than the CWRs likely as a result of breeding and domestication (Chapter 3)
- Seed morphology measurements such as mean seed mass and embryo length were correlated with thermal time for the *Hordeum* and *Brassica* genus. However, in the *Helianthus* genus, thermal time was correlated with the thickness of the seed coat relative to embryo length (Chapter 3, 4, 5 and 6)
- The crop seed lots of *Hordeum* and *Helianthus* germinated faster (on a thermal and hydro time basis) compared to their CWRs (Chapter 4 and 6), a likely consequence of breeding and domestication. However, seeds of the *Brassica* crop were not faster than those of the CWRs (Chapter 5).
- The CWRs are better equipped to cope with climate changes because of their capacity to develop normal seedlings at a wider range of conditions, probably due to their genetic variability and their exposure to the natural selection process (Chapter 4, 5 and 6)
- The germination parameter, thermal time, was influenced by the environment of the seed collection site including the mean monthly precipitation for *Brassica* CWRs (Chapter 5) and the annual mean temperature for *Hordeum* and *Helianthus* CWRs (Chapter 4 and 6).
- In general, the maternal environment of the CWRs (i.e., environment of the seed collection site) had more influence on seed functional traits than the progeny environment (i.e., predicted month of germination).

- The effect of the water treatments applied to the mother plant during seed filling on crop seed lots had significant effect in a few seed functional traits, but it was not consistent between seed lots.
- Seed functional traits such as thermal and hydro time and their thresholds are proposed as descriptors of seed vigour due to the comprehensive definition they provide for a seed population: T_b, T_c and Ψ_b describe the thresholds of a seed lot (Chapter 4, 5 and 6) and thermal and hydro time described seed germination rate under two main environmental factors, temperature and water. In addition to germination rate, thermal and hydro time change predictably as viability decreases in *Helianthus annuus* seeds stored under a wide range conditions (Chapter 7).

These main findings will be discussed in turn to understand their significance.

8.2 Breeding and domestication in *Hordeum*, *Brassica* and *Helianthus*

The practice of agriculture started with the sowing of a vigorous seed to provide plants and food for humans or animals. One of the earliest consequences of domestication was the increase in seed size (Meyer *et al.*, 2012). Although the selection of CWRs was limited in this thesis, in general, all the crop seeds showed greater mass than the CWRs seeds resulting from years of domestication (*c.* 6-fold in *Hordeum*, 2-fold in *Brassica* and 18-fold in *Helianthus*, Chapter 3). These increases in mass were reflected in increased storage tissues for *Hordeum* and *Brassica* and in the seed oil content for *Helianthus* crops as observed in Chapter 3. Thus, crop selection has focussed on improving yield, through the grain of barley seeds and oil of sunflower and brassica seeds. In future studies, a broader selection of CWRs, including wild *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus*, will strengthen these results. Greater seed mass was associated with longer embryos in the *Hordeum* and *Brassica* genus with opposite responses in relation to the θ_T. Seeds with longer embryos germinated faster (shorter θ_T) in the *Hordeum* genus, but slower in the *Brassica* genus (Chapter 4 and 5 respectively). The trend found in the

Hordeum genus is generally the case for other species. Seeds of the *Brassica* genus with short embryos had thin seed coats and this may contribute to fast imbibition. The lack of correlation, in the *Helianthus* genus, between the embryo length and germination rate could be due to the presence of the pericarp. The pericarp is thicker than the seed coat of *Hordeum* and *Brassica* seeds and it has a principal role in seed germination as observed in *Helianthus* CWRs. In future studies, *Helianthus* seeds without the pericarps could be germinated. The germination rate will be monitored and compared with the embryo length to observe whether there is a correlation between them.

Crop species have been selected to germinate faster under optimal conditions, thus it was hypothesised that crop seeds will have shorter thermal and hydro times compared to the CWRs (Dürr *et al.*, 2015). This was generally the case in the crops studied here. Faster germination is usually beneficial in environments with periods of scarce precipitation. Seeds will germinate after a rainfall event and establish and develop fast before the soil dries. Thus, rapid seedling establishment may ensure the survival of a plant by avoiding drought events in an early plant lifecycle stage. The exception was in the *Brassica* genus where domestication and breeding was not reflected in the germination rate of the crop seed lots which did not differ significantly from the CWRs, when germinated under the same conditions.

Crops are usually grown under managed conditions (i.e. closer to "optimal conditions") where they show the best performance. Thus, it is expected that seed crops outside of this "optimal" environment will struggle to survive. This was the case when the crops were germinated at low water potentials. Seeds of *Brassica* CWRs exhibit a higher tolerance to low water potentials than the crop seed lots (*c.* 2-fold lower Ψ_b on average). This suggests that seeds of the *Brassica* genus have sufficient genetic resources to "adapt" to environments with limited water. However, the Ψ_b of the *Hordeum* CWRs and crop seed lots did not differ, both having a similar tolerance to low water potentials. On the other hand, the crop genotypes of *Helianthus annuus* had lower Ψ_b than the perennial and dormant CWRs. Only the annual and non-dormant CWR, *H. argophyllus*,

exhibited a higher tolerance to low water potentials and thus could be a useful candidate to improve seed tolerance to low water potentials in *H. annuus*.

The presence of GA_3 (used to break seed dormancy on perennial *Helianthus* CWRs) during seed germination with PEG solutions (used to simulate drought) could disguise the real tolerance to low water potentials (Ni & Bradford, 1993; Alvarado & Bradford, 2005) of the dormant *Helianthus* CWRs. In future experiments Ψ_b of the perennial *Helianthus* CWRs seeds should be explored after seed dormancy has been alleviated by other methods that do not interact with PEG. This includes methods such as cold or warm stratification (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006).

Of the species studied here, five seed lots of *Hordeum* were from the European clade; two of *H. bulbosum* and *H. murinum* and one of *H. marinum*. Unfortunately, only one seed lot of the American clade was available, *H. pusillum*. Additionally, four seed lots of perennial *Helianthus* CWRs were studied, *H. pumilus, H. glaucophyllus* and two seed lots of *H. angustifolius*. Only one annual species was used, *H. argophyllus*. Therefore, in future studies, it is recommended that further seed lots from the American clade in the *Hordeum* genus and annual seed lots of *Helianthus* will strengthen the trends identified in seed functional traits of *Helianthus* and *Hordeum* CWRs.

The importance of historical georeferenced collections of CWRs has been highlighted here as well as the important role of seed banks. Plant professionals started a collection of species to conserve *ex situ* Plant Genetic Resources for Food and Agriculture (PGRFA). Nowadays, 7.4 million PGRFA accessions are available worldwide. CWRs are still underrepresented (FAO, 2010; Castañeda-Álvarez *et al.*, 2016) although the Crop Trust and the Millennium Seed Bank (MSB) of the Royal Botanic Gardens, Kew (UK) are studying retained functional traits are reflective of their maternal environment. The MSB has seeds of over 37,000 wild plant species from across the world, from which over 300 are CWRs. I found that seeds of CWRs stored up to 20 years in the MSB were of high seed viability (> 80 %). Thus, seed banks are an important source of functional

traits of CWRs available for the future and their representation in *ex situ* collections needs improving. Timme *et al.* (2007) concluded that the *Helianthus* genus consists of 49 species (37 perennial and 12 annual) of which there are 28 species of wild *Helianthus* in the MSB. Six of these are annual CWRs (*H. annuus, H. anomalus, H. argophyllus, H. bolanderi, H. deserticola* and *H. petiolaris*). Nonetheless, only one accession of annual species, *H. argophyllus,* had enough seeds available to use for research in this thesis. It is important therefore to define seed numbers for CWR banking and use, separate to that of conservation. Future recommendation to increase the presence of CWRs in *ex situ* conservation programmes are included in section 8.7.

8.3 Are crop seeds relatively dysfunctional?

Seed vigour has been widely described as a complex trait that will include several properties such as homogeneous and fast germination and normal seedling development under a wide range of environments (Pollock & Roos, 1972; Rajjou *et al.*, 2012; Finch-Savage & Bassel, 2015; ISTA, 2017). In the seed germination experiments carried out, such as high temperatures or low water potentials (e.g., *Hordeum vulgare* at 35 ºC, *Brassica oleracea* at 35 ºC and -1.0 MPa and *Helianthus annuus* at 30 and 35 ºC), a germinated seed did not always convert into a normal seedling. Thus, normal seedlings of the CWRs were compared with their crops to assess conversion success and general vigour (Chapter 4, 5 and 6). In this respect, CWRs showed higher vigour (greater normal seedling conversion) than crop seed lots under the same controlled germination conditions, especially at higher temperatures and lower water potentials. Thus, it is important to fully characterise the thermal-hydro dependency of both germination and subsequent seedling development.

Of the three crop genera explored in this project, *Helianthus annuus* seedlings were the most vulnerable to high temperatures and low water potentials compared to *Hordeum vulgare* and *Brassica oleracea*. Therefore, on extreme climate events such as drought or heat stress, the vigour of the crop seed lots is likely to be lower compared to the vigour of CWRs (Chapter 6). This finding implies an unintended deleterious

consequence of breeding and selection of the crop seed lots from growth under managed conditions. The crop seed lots may have lost adaptability to the supra-optimal range of temperatures during the domestication and selection process. For example, at 25 ºC and 30 ºC less than half of the seeds developed into normal seedlings. In this case, there is potential to improve the conversion into normal seedlings to those temperatures through the selection in plant breeding of seedlings that survived. On the other hand, at 35 ºC there was no normal seedling growth, i.e., no survival in any crop seed lot. Suggestions for crop improvement in terms of survival of normal seedlings are included in section 8.7.

8.4 Influence of the maternal and progeny environment in population-based model (thermal and hydro time and the thresholds) under controlled conditions and their application to the field

Germination traits were described using thermal and hydro time models that have been widely used to quantify seed germination progress and seedling emergence of a seed lot (Bradford, 2002; Finch-Savage, 2004). The base temperature, T_b, did not differ significantly among crop genotypes in *Brassica oleracea* (low and high vigour) and *Helianthus annuus* (genotypes A, B, C, D and E). In both of these genera, the plants of the crop genotypes were grown under the same glasshouse conditions or in the same field at the same time respectively. Similarly, Ellis *et al.* (1986) described the same T_b among different crop genotypes of chickpea. T_b did not appear to be influenced by genetics. In contrast, for the seed lots of the CWRs where the same species were collected from two different environments, T_b differed among them (e.g., *Hordeum murinum* Table 4.1, *Brassica rapa,* Table 5.1, *Helianthus angustifolius* Table 6.1). Thus, the T_b seems to be influenced by the environment as demonstrated here in the genotypes of the crops (same species growing under the same environments) and the seed lots of the same CWR (same species growing under different environments). However, it is important to consider the genotype x environment interaction and a recommendation is made in section 8.7.1.

The germination parameters, T_b, Ψ_b, θ_T, θ_H and θ_{HT}, can be used to find ecological correlations with the maternal environment (Benech Arnold *et al.*, 1990; Allen *et al.*, 2000; Forcella *et al.*, 2000; Bradford, 2002; Murdoch & Kebreab, 2013). In crop species, Finch-Savage et al. (1998) showed that a modification of θ_{HT} model reliably described seed germination performance of carrot seeds in the field. Porceddu *et al.* (2013) showed that their estimations of θ_T values under controlled conditions allowed them to predict the time of emergence in the field by *in situ* observations in one endemic tree in Mediterranean climates. Kebreab & Murdoch (1999a) used a modification of the θ_T to estimate the emergence of a parasitic weed of sunflower (*Orobranche* spp.) to improve the sowing strategies for future control on crops. Furthermore, these models are also used to predict seed germination in future climate scenarios (Grundy *et al.*, 2000; Grundy, 2003; Cochrane *et al.*, 2014a). For example, Orrù *et al.* (2012) combined the temperature of the environment (mean monthly temperature) with the θ_T values to predict germination under different future climate scenarios

Consistent with such studies that successfully link germination parameters to the environment, in this thesis the maternal and progeny environment (of seed collection site) of the CWRs were correlated with θ_T. Specifically, the historical annual mean air temperature (minimum, mean and maximum of the maternal environment) in *Helianthus* CWRs. The seeds of *Helianthus* CWRs produced at warmer temperatures had improved germination rate (shorter θ_T) compared to seeds produced at cooler temperatures. Similarly, seeds of several species in which the maternal environment had higher temperatures had higher germinability, lower dormancy level and faster germination than seeds produced at lower temperatures (Guisan & Zimmermann, 2000). Therefore, warmer temperatures in the maternal environment could be applied in the production of *H. annuus* seeds to increase the speed of germination on a thermal basis.

On the other hand, the seeds of *Hordeum* CWRs germinated slower (longer θ_T) at warmer temperatures than at cooler temperatures of the

progeny environment. This could be a strategy to avoid exposing the seedlings to high temperatures.

In contrast, θ_T of the *Brassica* CWRs seeds was correlated with the mean monthly precipitation. In this case, the presence of water was the main factor that influenced germination rate on a thermal basis. *Brassica* CWRs usually grow in Mediterranean climates where seeds may be adapted to germinate fast to avoid drought periods during seedling growth.

Additionally, seed functional traits of CWRs such as germination rate (θ_T and θ_H), seed germination thresholds (T_b and Ψ_b), embryo length and seed mass appear to be more influenced by the maternal environment (i.e., environment of seed collection site) than the progeny environment (i.e., predicted month of germination). Thus, the knowledge of the environment of the mother plant is of great importance to understand the performance of a seed population because of its influence on seed functional traits.

The effect of the irrigation treatments applied to the mother plants during seed filling for one generation in the crop genotypes of *Hordeum vulgare, Brassica oleracea* and *Helianthus annuus* did not have a single predictable impact. In the case of *Helianthus annuus*, the stopped irrigation treatment had no consistent effect amongst the five Limagrain genotypes. Moreover, the irrigation treatment did not affect seed longevity since both seed lots, normal and stopped irrigation, aged at the same rate (Chapter 7). These genotypes had different flowering times (Chapter 2, Table 2.5) and the stopped irrigation treatment was applied at the same period (end of July to the beginning of September). Hence, the stopped irrigation treatment was probably not applied at the same physiological stage for all Limagrain genotypes. Moreover, the plants were grown in the field where further environmental factors could interact with the irrigation treatment. In contrast, the mother plants of *Brassica oleracea* were grown under controlled conditions in a glasshouse. The irrigation treatment did not affect seed germination traits. However, the plants that grew under "drought treatment" produced seeds with thicker seed coats in both genotypes, low and high vigour (A12DHd and AGSL101 respectively). Thus, seed coat thickness shows plasticity in *Brassica oleracea* when the mother plant is subjected to water limitations during the seed filling stage.

Thicker seed coats may provide a better physical defence when facing adverse biotic or abiotic conditions (Mohamed-Yasseen *et al.*, 1994). Finally, plants of *Hordeum vulgare* subjected to water limitation treatment during seed filling produced seeds with more tolerance to low water potentials (i.e., lower Ψ_b). This could be described as an adaptation to "water stress" of the seeds acquired from the mother plant when dealing with water limitation. To summarise, it seems that *Hordeum vulgare* seed lots appear to have sufficient plasticity that tolerance to low water potential could be enhanced through selection in plant breeding. In contrast, in the other two genera the plasticity of the seeds to low water potentials is less predictable.

In this thesis, the interactions between temperature and water potentials were not considered because the germination experiments at low water potentials were performed at only one temperature (due to seed availability). However, as mentioned previously, a modification of the hydrothermal time model considers the interaction between temperature and water potential (Benech-Arnold *et al.,* 1990, Forcella *et al.,* 2000, Roman *et al.,* 2000). In future studies, the germination experiment at different water potentials should be performed at several temperatures to observe the interaction between them as Kebreab & Murdoch (1999b) showed. Ψ_b can be obtained for each temperature and afterwards the hydrothermal time across all the temperatures tested. Regarding the interactions between temperature and water potentials

In addition to predicting seed germination in the field, these models can also be associated with other physiological processes such as seed longevity (Chapter 7, Bradford *et al.,* 1993). Therefore, the seed traits and model parameters have relevance in agriculture to predict seed quality and performance. Seed companies assess seed vigour by germinating seeds under controlled conditions using a single temperature, but the seeds of different species may have different thermal thresholds and germination requirements. Thus, greater attention needs to be given to establishing the germination thresholds of a seed lot. In addition to calculating seed germination thresholds and thermal time under controlled conditions, future work should validate the models of CWRs in the field. This can

contribute to solve the complex seed functional traits x environment interaction.

The findings of this study may have practical implications on seed industry by providing insights into seed functional traits of CWRs. This research has also contributed to the EcoSeed project; an EU funded project whose main goal was trying to solve problems related to seed quality and storability. My investigation of seed functional traits and the impact of the maternal environment (temperature and water) provided phenotypic comparisons between the crops and their CWRs, supporting biochemical and molecular studies of the project partners. The research highlighted possible genetic sources of CWRs as well as validating markers of seed quality that were developed for crops. The findings obtained by studying CWRs on the EcoSeed project can be transferred into agriculture and conservation.

8.5 Seed functional traits as descriptors of seed vigour

Vigour can be defined as the combination of seed characteristics that determine the potential level of performance and fitness of the seed during germination and seedling development under both favourable and adverse conditions, including the capacity to germinate after storage (Finch-Savage & Bassel, 2015). Differences in seed vigour can be incurred due to genetic differences between two seed lots, for example as observed in the genotype A12DHd and AGSL101 of *Brassica oleracea* or differences in their initial viability affecting the field emergence in adverse or favourable conditions (Khah *et al.,* 1986). Physiological vigour can also show differences between seed lots with the same genetic background as observed in the water treatments applied in the crop research genotypes of *Hordeum, Brassica* and *Helianthus*. The possible interactions between the environment and vigour are enormous because it includes many factors such as water limitations and temperature fluctuations (Pollock & Roos, 1972; Finch-Savage & Bassel, 2015). For these reasons, I used the combination of seed functional traits, thermal and hydro time and their thresholds and seed longevity as precise descriptors of seed vigour. The

determination of these traits defines the seed lots in response to a wide range of two main environmental factors, temperature and water. The thresholds T_b, T_c and Ψ_b illustrate the limits of the seed lot describing the range of temperatures and water potentials where the seeds can germinate. In addition to indicating seed germination rate at several temperatures and water potentials, θ_T and θ_H have been found to be associated with seed viability levels imposed by a wide range of storage conditions (Chapter 7).

The findings it Chapter 7 are the first attempt to fully quantify seed vigour by combining the characterisation of thermal and hydro time parameters of seed germination and seed viability during ageing in sunflower seeds. In this study, they were calculated from radicle emergence records (seed germination). However, future studies should also cover the analysis of θ_T, T_b, T_c, θ_H and Ψ_b based on the quantification of normal seedlings under each germination conditions for any species explored. For example, in cases such as *Helianthus*, an increase of 5 °C (above 25 ºC) under controlled conditions will shift the species out of their optimal range for normal seedling growth.

8.6 Are CWRs necessary for breeding? Advantages and disadvantages

Nowadays most crops have been improved to obtain higher yields (Porter & Semenov, 2005). However, in some crops such as wheat, the increases in yield have been stopped lately due to warming temperatures in Europe (IPCC, 2013). Recently, physiological phenotyping to identify and select desirable traits, such as drought tolerance, have increased (Fiorani & Schurr, 2013; Ghanem *et al.*, 2015).

The effect of climate change is likely to affect crop yields and plant survival in the field. Although sowing time in the field can be changed to avoid stresses, different sow timings (earlier or later) could be applied to modify the timing of further plant development stages (Donohue *et al.,* 2015) to avoid drought periods or improve fitness (Franks *et al.,* 2011). Thus, it is necessary to explore how crop seeds will respond under non-optimal or managed conditions to ensure its survival and persistence in several locations.

CWRs are a natural and important source of genetic diversity that is used to improve crops for desirable resistance and tolerance to several stresses. In some cases, there is a barrier of incompatibility between CWRs and crops such that their cross-pollination results in sterile plants or non-viable seeds. In these cases, the process of introducing a desirable trait to crop plants can be difficult, slow and expensive. However, the pressure to enhance agricultural production and create new cultivars or varieties to cultivate crops in extreme environments (Ford-Lloyd *et al.*, 2011) such as locations with high temperatures or scarce water availability, degraded soils (i.e., poor in nutrients) or soils with high salt content, is increasing due to climate change. Thus, finding CWRs with desirable traits is of great interest and of huge potential benefit to agriculture over the longer-term.

The crop *Hordeum vulgare* has compatibility with its progenitor *H. vulgare* ssp. *spontaneum* and hence, it has been widely used in breeding programs. *H. bulbosum* is the second species most used to improve *H. vulgare* since the cross-pollination between them can produce fertile seeds (Morrell & Clegg, 2011). However, there are other CWRs such as *H. marinum* that possess desirable traits (e.g. salt tolerance) (Alamri *et al.*, 2013), to use in breeding programs. In this thesis it was found that the crop seeds did not have the capacity to develop normal seedlings at -1.0 MPa (Chapter 4, Table 4.2). In contrast, the seeds of *Hordeum* CWRs studied here, were able to develop normal seedlings at -1.0 MPa. Thus, *Hordeum* CWRs could be used to improve crop seedlings growth under conditions of low water potential.

The species *Brassica oleracea* forms several varieties of crops with different morphology and uses for human consumption (i.e., kale, broccoli, cabbage or Brussels sprouts). CWRs of *B. oleracea* are widespread in Europe and are used for improving the crop. In this thesis, the seed germination analyses found possible opportunities to improve *Brassica* crop seed lots such as the lack of normal seedlings development at low water potentials (-1.0 MPa). *Brassica* CWR seeds were capable of developing into normal seedlings under those conditions. Moreover, *Brassica* CWRs, in particular *B. rapa* seed lots, had lower Ψ_b than the crop seed lots (Chapter 5, Table 5.1) which was not observed in *Helianthus* nor

Hordeum CWRs when comparing with their crops. Therefore, a better understanding of the factors controlling CWRs seed traits could be used to improve crop varieties with the capacity to grow and survive under limited water environments.

Breeding of the crop *Helianthus annuus* is usually achieved using annual *Helianthus* CWRs (Seiler & Gulya, 2004). However, the perennial *Helianthus* CWRs are a great source of genetic diversity, but due to seed dormancy or sexual incompatibility producing fertile and non-dormant seeds is a challenge (Seiler *et al.*, 2017). In the *Helianthus* genus, the most important problem found in this thesis was the incapacity of crop seeds to develop into normal seedlings at higher temperatures (Chapter 6, Figure 6.3 and 6.4). Despite the fact that germination percentage was no higher than 50 % (Chapter 6, Figure 6.2), most of the seeds of perennial *Helianthus* CWRs had the capacity to produce normal seedlings at 30 and 35 ºC. Thus, the characterisation provided here of seedling survival of *Helianthus* CWRs at high temperatures could be used to improve the growth of normal seedlings at high temperatures in the crop, *H. annuus*.

In summary, the use of CWRs in breeding programs is of paramount importance to improve some aspects of their respective crops. Progress may depend on the new methods that have been developed to overcome problems of incompatibility. For example, the introgression of desirable traits by using "bridge species" that are compatible with both the crop and the CWRs that possess the desirable trait (e.g., the result of crossing *Hordeum chilense* and *Triticeae* crops is used to improve wheat cultivars, Martín *et al.,* 1999).

8.6.1. Seed priming as an alternative to improve low vigour

Seed vigour can be improved through breeding and using key traits from CWRs, but this technology is still unavailable for others than large breeding companies. Nonetheless, breeding is not the only known technique to improve seed vigour. Seed priming (pre-germinative treatments) has been proved to improve seed vigour and it is a low-cost alternative that farmers could use to improve the germinability of their crops (i.e., germination rate and uniform seedling emergence) (Blocklehurst & Dearman, 1983, Harris 1996). Seeds required different

priming duration or techniques (soaking seeds in water or chemical solutions, cold or warm temperature) depending on the type of crops (i.e., Harris *et al.,* 1999, Harris *et al.,* 2002, Dahal *et al.,* 1990 and Aldergerich *et al.,* 1989).

Even though priming can be an interesting tool to improve seed vigour of many crops, it is still necessary to investigate the best priming technique for each crop. Furthermore, low vigour of a seed lot can be exhibit under several stresses such as drought, salt or heat. In those cases, seed priming might not be sufficient to improve vigour and then breeding would be necessary to obtain seed lots with drought resilience, salt tolerance or heat tolerance.

8.7 Recommendation for future work

8.7.1 Genotype x environment interaction.

In this thesis it was not possible to distinguish between genotype or environmental effects nor their interaction on the seed populations behaviour. In future experiments, the use of a common garden to observe the genetic differences of seed functional traits of the populations is highly recommended (as the Limagrain genotypes used here). Moreover, the possibility of placing the same genotypes in several environments will provide the environmental effects on seed functional traits and it will be possible to distinguish between genotype and environmental effects in addition to their interaction.

8.7.2 Improve the plasticity of seed functional traits of crops.

Problems of the capacity to develop normal seedlings on crop seed lots at higher temperatures and low water potentials, have been discussed here. In future breeding programmes, crops will need to be bred for robustness to face changes in the environment by increasing phenotypic plasticity in seed traits such as germination and seedling normality to obtain resilience to high temperatures and low water potentials. A balance between germination rate and normal seedling growth should be found across a large range of environments. In other words, the range of optimal temperatures for crops should be the range that has fast and uniform

germination and greatest conversion into normal seedlings (100% of the germinated seeds develop into normal seedlings). This could be achieved by selecting individuals of the population that have the capacity to convert into normal seedlings under non-optimal conditions.

An alternative approach to this problem is to exploit CWRs in which seedlings grow under more extreme conditions, such as *Brassica rapa* that produced normal seedlings at -1.0 MPa or *Helianthus angustifolius* that produce normal seedlings at 25 and 30 and 35 ºC. This ability of CWRs was identify in this thesis, and could be transferred, through breeding programmes, to their respective crops to obtain the capacity of seedling survival under those conditions.

8.7.3 Validate seed germination models in the field.

Field observations are useful to provide knowledge of the emergence of wild species and understand the physiological process in the wild. However empirical seed germination models, under controlled conditions, offer a quick and simple indication of great importance when the fieldwork is not possible, such as predictions in future climate scenarios. Moreover, the application of the seed germination models under controlled conditions used here, has been previously validated in the nature (Benech Arnold *et al.*, 1990; Forcella *et al.*, 2000; Eizenberg *et al.*, 2012).

Seed traits such as seed dormancy make the prediction of seed germination and emergence in the field difficult. Thus, the best model should be obtained from a combination of both, controlled conditions and field conditions especially in wild species with different characteristics such as seed dormancy.

Another valuable application of the models under controlled conditions is the characterisation of the seed lot under a wide range of "environments" when the environment of the field is not manageable. The characterisation of the Ψ_b of a seed lot is usually measured at optimal conditions, however Ψ_b varies with temperature. For example, Ψ_b values of seeds germinated at warm temperatures (above T_o) tend to be higher (lower tolerance) than Ψ_b of seeds germinated at T_o or below. Therefore, Ψ_b should be characterised at several temperatures (sub- and supra-optimal temperatures) obtaining

an accurate germination threshold to avoid water stress. In this manner, a wider description of the seed lot fitness will be obtained to relate with several environmental scenarios to cover the geographic limits of germination.

8.7.4 Increase representation of CWRs in *ex situ* conservation

There is a concern of the poor representation of CWRs in gene banks. Firstly, a broad study identifying gaps of the presence of CWRs in *ex situ* conservation is needed. Then, future studies should prioritise CWRs from habitats that are threatened by changes in the environment (natural or human activities) where *in situ* conservation will not be possible, and particularly areas with the greatest potential sources of genetic diversity. Finally, international collaboration between organisations and countries is of paramount importance to share knowledge, have duplicates of *ex situ* conservation, obtain funds that could be destined to optimise, improve and maintain *in situ* and *ex situ* conservation of CWRs and other recognised useful species.

8.8 Conclusions

In agriculture CWRs play an important role in breeding as resources of variability and resilient genes to biotic and abiotic stress. Therefore, they should be preserved as an essential source of seed functional traits. These traits need to be described and understood with the objective to enhance seed vigour of important crop species in future climate scenarios. The characterisation and comparison of seed functional traits of CWRs and crops performed here, has revealed some limitations to face future environmental changes. Future approaches should consider crop breeding programmes to improve crop resilience as well as investigations to optimise seed production and storage of the CWRs.

The quantification of seed functional traits in CWRs obtained in this thesis shows the merits of this approach which should be extended in order to help the improvement of agriculture production. This knowledge may be transferred to seed conservation and the seed industry to help ensure the persistence of wild species and sustainability of agriculture respectively.

Plant regeneration depends on seed germination and thus seed germination thresholds obtained in the species studied here could be used as a guidance when facing future climate scenarios. Subsequent life stages (i.e., flowering time and seed filling) are determined by the timing of germination (when both stages are under the same environment) based on the thresholds when further development does not occur (Donohue *et al.*, 2015). Therefore, quantifying seed germination traits will contribute to understanding how environmental changes may influence plant regeneration.

9 APPENDIX

9.1 Appendix Chapter 2

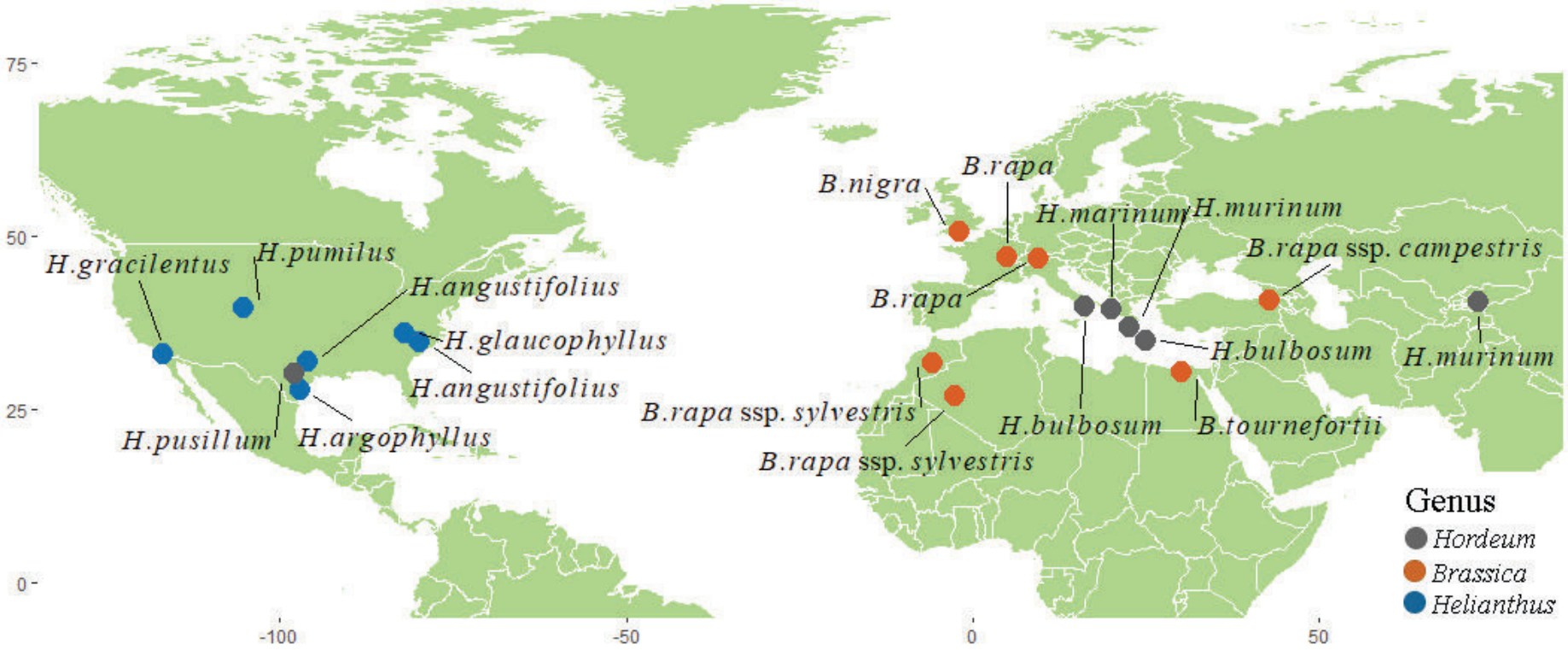


Figure A2.1 Distribution of the CWRs used in this thesis provided by the Royal Botanic Gardens, Millennium Seed Bank, Kew (UK) from three genera, *Hordeum* (grey), *Brassica* (orange) and *Helianthus* (blue).

9.2 Appendix Chapter 3

Table A3.1 Temperature at which the germination percentage was highest. The germination percentage (G) is the mean of three replicates for CWRs and four replicates for crops at the specified temperatures (T °C).

CWRs	G (%)	T (°C)
Hordeum marinum (Greece)	100 a	15
H. bulbosum (Italy)	93.2 b	5
H. pusillum (USA)	83.9 c	10
H. bulbosum (Greece)	93.3 b	5, 10, 15
H. murinum (Greece)	100 a	20
H. murinum (Kyrgyzstan)	100 a	10, 15
Brassica rapa (Switzerland)	93.3 a	20
B. nigra (England)	94.7 a	25
B. rapa (France)	92.0 a	20
B. rapa subsp. *campestris* (Turkey)	98.7 a	25, 30
B. rapa subsp. *sylvestris* (Morocco)	100 a	15, 20, 25
B. tournefortii (Egypt)	97.3 a	20, 25
B. rapa subsp. *sylvestris* (Algeria)	100 a	30
Helianthus glaucophyllus (North Carolina)	86.2 ab	10
H. angustifolius (North Carolina)	78.9 b	30
H. angustifolius (Texas)	89.5 ab	25
H. argophyllus (Texas)	100.0 a	10, 20
H. pumilus (Colorado)	81.9 ab	10

Table A3.1 Continuation of the Table

Crops	G (%)	T (°C)
Hordeum vulgare control treatment	100 a	5, 20, 25
H. vulgare drought treatment	100 a	5, 15, 20
H. vulgare commercial seed lot	100 a	15
Brassica oleracea A12DHd control treatment	100 a	20, 25
B. oleracea A12DHd drought treatment	99.0 a	10, 15, 25
B. oleracea AGSL101 control treatment	100 a	20, 25, 30
B. oleracea AGSL101 drought treatment	100 a	10, 20, 25
B. oleracea commercial seed lot	100 a	10, 20, 25, 30
Helianthus annuus A normal irrigation	94.7 ab	20
H. annuus A stopped irrigation	92.2 ab	20
H. annuus B normal irrigation	88.8 b	10, 20
H. annuus B stopped irrigation	98.0 ab	10, 20
H. annuus C normal irrigation	100.0 a	20
H. annuus C stopped irrigation	96.1 ab	10
H. annuus D normal irrigation	97.3 ab	10, 20
H. annuus D stopped irrigation	100.0 a	10
H. annuus E normal irrigation	100.0 a	10, 20
H. annuus E stopped irrigation	100.0 a	10, 20
H. annuus commercial seed lot	99.0 a	20

Table A3.2 Percentage of water uptake of five *Helianthus* CWRs in non-scarified (No-SC) and scarified (SC) seeds after 24 hours at 20 °C. The values are the mean of 15 seeds.

Species	% water uptake No-SC	% water uptake SC
H. glaucophyllus	54.99 ± 14.6	65.42 ± 21.0
H. angustifolius North Carolina	67.85 ± 18.6	87.78 ± 28.0
H. angustifolius Texas	59.05 ± 10.5	66.67 ± 12.2
H. argophyllus	28.33 ± 7.3	34.24 ± 5.3
H. pumilus	57.93 ± 11.5	64.42 ± 15.7

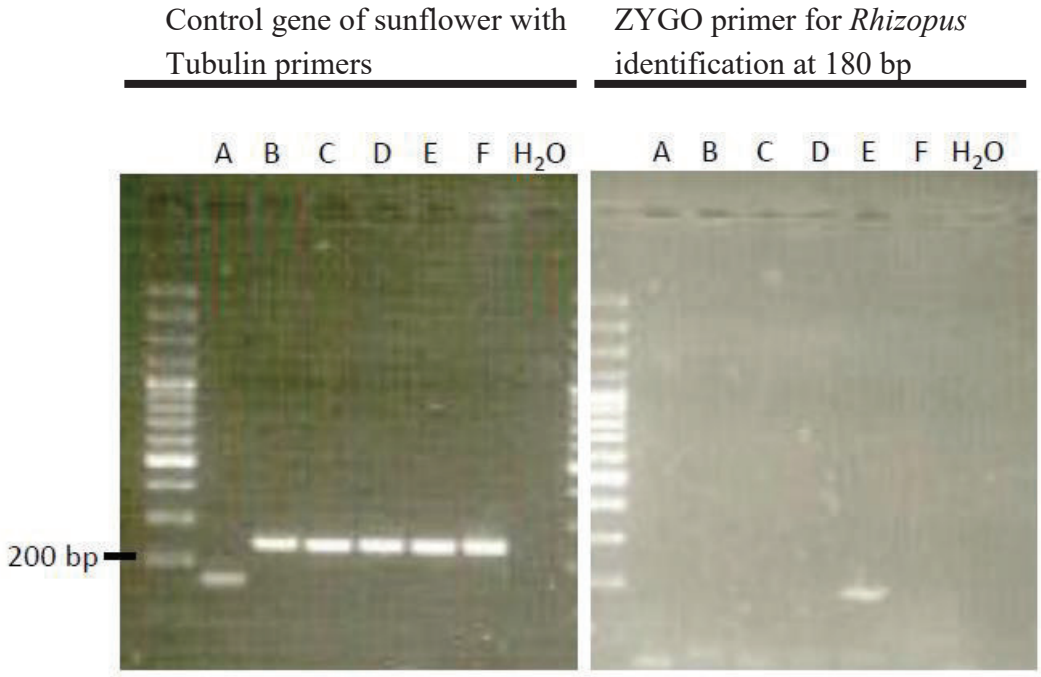


Figure A3.1 PCR of genotype B of *Helianthus annuus* DNA. The left image is a control with Tubulin primers to test PCR efficiency. In the right image ZYGO+/ZYGO- primers were used to identify fungi from *Rhizopus* genus. A is cDNA of sunflower seeds. B is the DNA of seeds from a different sunflower lot. C is the DNA of the cotyledons from non-infected seeds. D is the DNA of the axes from non-infected seeds. E is the DNA of the cotyledons from infected seeds and F is the DNA of axes from infected seeds. Image provided by the Université Pierre et Marie Curie, France.

Table A3.3 Values and standard error (SE) of the parameters of the regression lines from the Figures of Chapter 3.

		Value	SE
Figure 3.1	Intercept	0.0234	0.00331
	Slope	0.0026	5.245E-4
Figure 3.2A	Intercept	1.5500	0.75578
	Slope	2.2370	0.47462
Figure 3.2B	Intercept	1.0505	0.18658
	Slope	0.0294	0.00881
Figure 3.3	Intercept	148.8505	11.98578
	Slope	-66.8366	9.19775
Figure 3.4A Embryo	Intercept	1.2380	0.12423
	Slope	0.1900	0.04011
Figure 3.4A Testa	Intercept	0.0148	0.00441
	Slope	0.0072	0.00142
Figure 3.4B	Intercept	-0.0093	0.01551
	Slope	0.0250	0.00859
Figure 3.8	Intercept	61.6461	3.30298
	Slope	-14.7918	2.52382
Figure 3.9A MinT	Intercept	-6.7929	4.8713
	Slope	8.9004	2.66789
Figure 3.9A MeanT	Intercept	1.9437	4.10157
	Slope	7.5474	2.24633
Figure 3.9B MinT	Intercept	-9.2101	4.95769
	Slope	42.6643	11.38942
Figure 3.9B MeanT	Intercept	-0.3533	3.8227
	Slope	36.7780	8.78198
Figure 3.9B MaxT	Intercept	8.5035	3.34173
	Slope	30.8917	7.67705

Table A3.4 Correlations between seed morphology and oil content with the environment of the seed collection site of **seven** *Brassica* CWRs. DF = 5

*P < 0.05 **P < 0.01 ***P < 0.001	Annual Min T (°C)	Annual Mean T (°C)	Annual Max T (°C)	m.a.s.l (m)	Seed mass (mg)	Embryo length (mm)	Thickness seed coat (mm)	Oil content %
Mean Precipt. (mm)	NS	NS	-0.82*	NS	NS	NS	NS	NS
Annual Min T (°C)	-	0.97***	0.90**	NS	NS	NS	NS	NS
	Annual Mean T (°C)	-	0.98***	NS	NS	NS	NS	NS
		Annual Max T (°C)	-	NS	NS	NS	NS	NS
			m.a.s.l (m)	-	NS	NS	NS	NS
				Seed mass (mg)	-	NS	0.88**	NS
					Embryo length (mm)	-	NS	NS
						Thickness seed coat (mm)	-	NS

9.3 Appendix Chapter 4

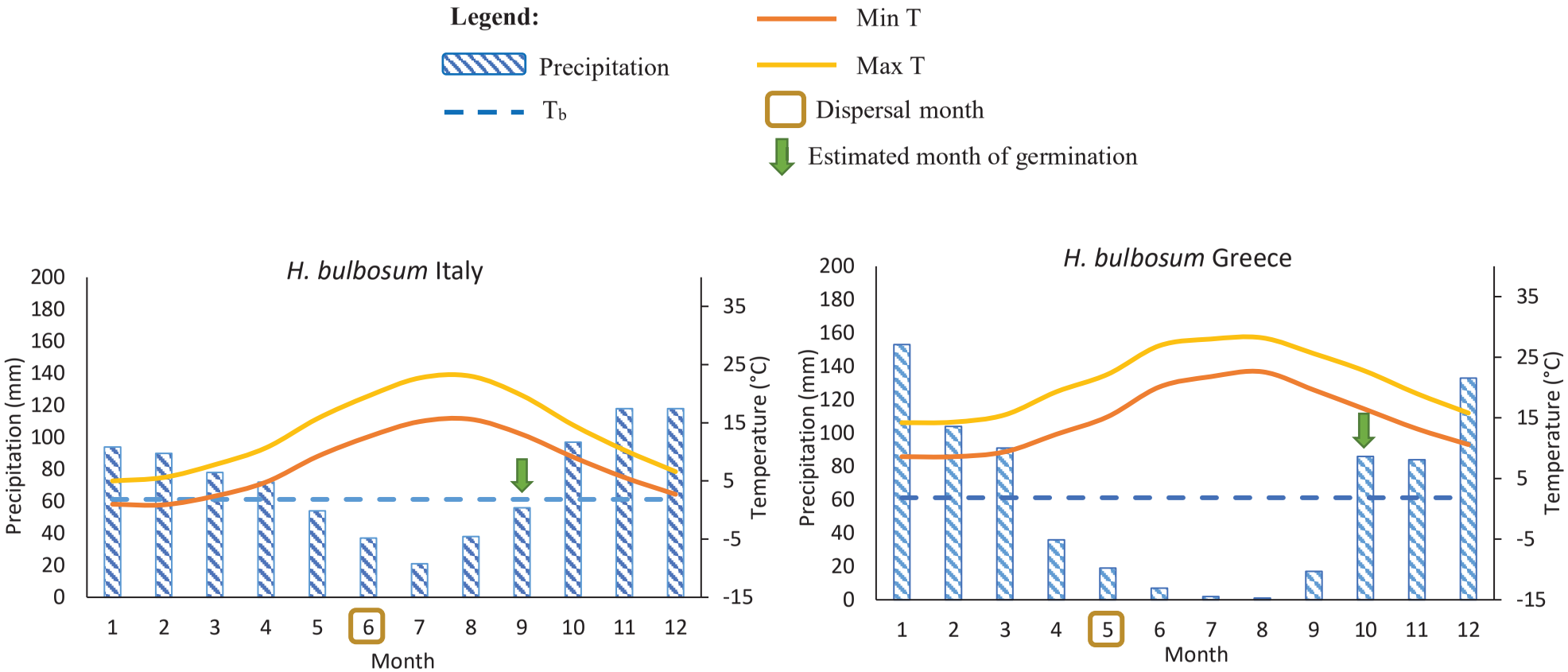


Figure A4.1 Historical annual mean temperature (minimum in red and maximum in yellow) and mean monthly precipitation (blue bars) in the environment of seed collection site for the six CWRs of *Hordeum*. The month enclosed in the orange square symbol represents the seed dispersal month according to the collectors. The green arrows are the estimated month of germination (MoG) when the seeds would be able to germinate. Two assumptions are made (1) the precipitation was above 15 mm, previous studies suggested this quantity (between 10 and 20) as the minimum rainfall the seeds were able to start the germination (Freas and Kemp, 1983, Gutterman, 1993, Gutterman, 2000a), and (2) the minimum environmental temperature exceeded T_b.

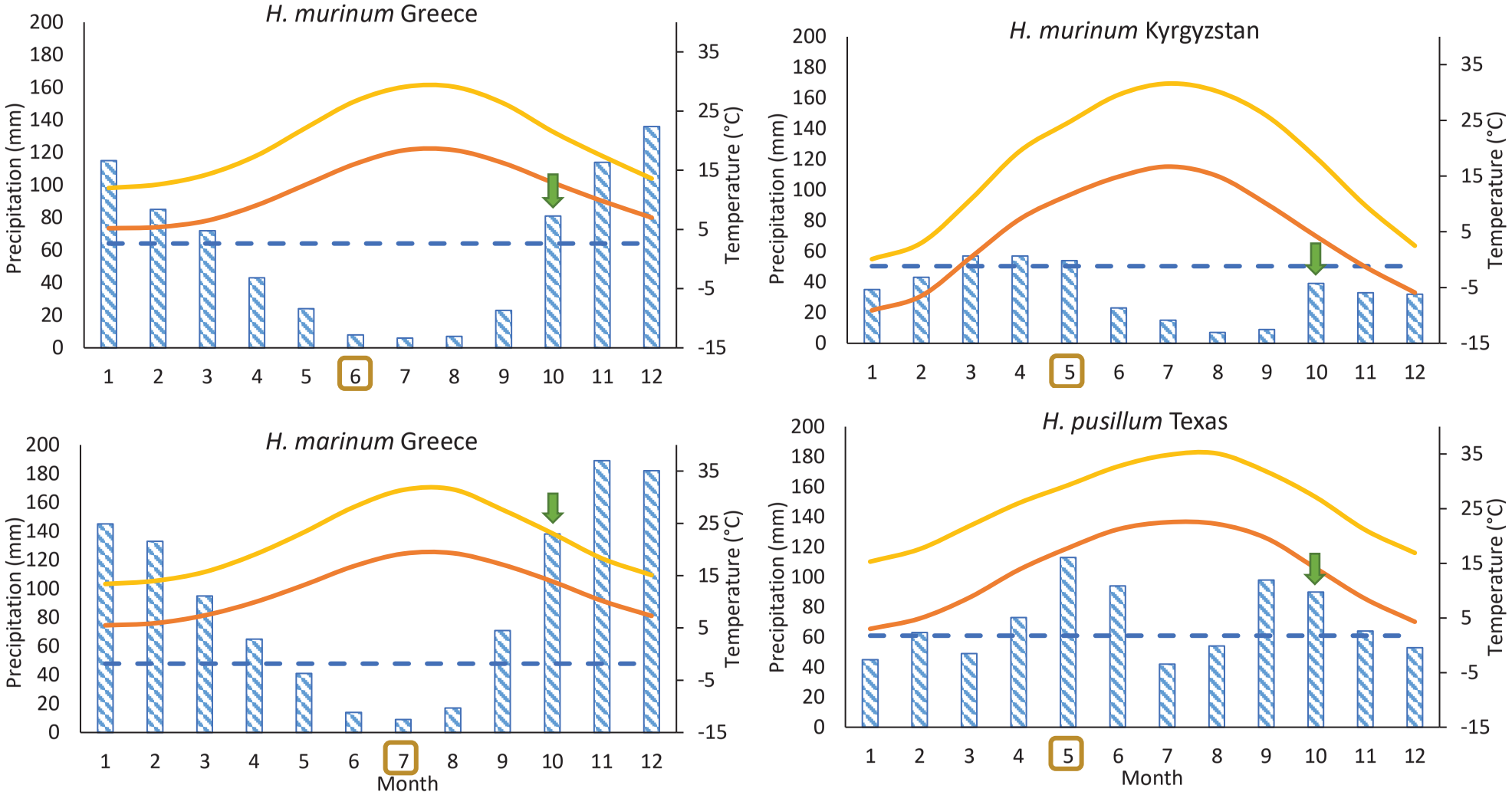


Figure A4.1 Continuation

Table A4.1 Table of correlations between physical and physiological seed traits of the *Hordeum* genus. Correlation coefficient values (r) for the linear correlations between seed morphology factors (embryo length, endosperm length, thickness of the seed coat and seed mass), the coefficient of variation (CV) of each seed morphology factor, and seed germination parameters (thermal time, θ_T, hydro time, θ_H, base temperature, T_b, base water potential, Ψ_b) of nine *Hordeum* seed lots (CWRs and crops). NS = Not Significant *P < 0.05; **P < 0.01; ***P < 0.001

	Embryo length (mm)	CV embryo	Endosperm length (mm)	CV endosperm	Thick Seed coat (mm)	CV seed coat	Viability (%)	Seed mass (mg)	CV Seed mass	θ_T (°Ch)	T_b (°C)	θ_H (MPah)	Ψ_b (MPa)
Embryo length	-	NS	0.85 **	-0.79 *	NS	NS	0.71 *	0.77 *	NS	-0.81 ***	NS	0.90 ***	NS
	CV embryo	-	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
		Endosperm length	-	NS	0.69 *	NS	NS	NS	NS	-0.85 ***	NS	0.84 ***	NS
			CV endosperm	-	NS	NS	-0.68 *	-0.72 *	NS	0.76 ***	NS	0.90 ***	NS
				Thick Seed coat (mm)	-	0.81 **	NS	NS	NS	-0.40 *	NS	-0.39 *	NS
					CV seed coat	-	NS	NS	NS	NS	NS	NS	NS
						Viability (%)	-	NS	NS	-0.72 ***	-0.41 *	-0.72 ***	NS
							Seed mass (mg)	NS	NS	**-0.51** **	-0.43 *	**-0.70** ***	NS
								CV Seed mass		NS	NS	NS	NS
									θ_T (°Ch)	-	NS	0.87 ***	0.38 *
										T_b (°C)	-	NS	0.51 **
											θ_H (MPah)	-	NS

Table A4.2 Table of correlations between seed germination traits with the environment of the seed collection site of **six** *Hordeum* CWRs. Correlation coefficient values (r) for the linear correlations between seed germination parameters (thermal time, θ_T, hydro time, θ_H, base temperature, T_b, base water potential, Ψ_b) and the maternal environment (annual mean and mean of the month of germination, MoG). In general DF = 4 except for #DF = 16. NS = Not Significant *P < 0.05; **P < 0.01; ***P < 0.001.

***P < 0.05 **P < 0.01 ***P < 0.001**	**Annual Min T (°C)**	**Annual Mean T (°C)**	**Annual Max T (°C)**	**m.a.s.l (m)**	**Precipit. MoG (mm)**	**MinT MoG (°C)**	**MeanT MoG (°C)**	**MaxT MoG (°C)**	**#T_b (°C)**	**#θ_T (°Ch)**	**#Ψ_b (MPa)**	**#θ_H (MPah)**
Mean Precipt. (mm)	NS	NS	NS	NS	0.82*	NS	NS	NS	NS	NS	NS	NS
Annual Min T (°C)	-	0.93**	NS	-0.95**	NS	0.88*	0.94**	NS	NS	NS	NS	NS
	Annual Mean T (°C)	-	0.94**	-0.91*	NS	NS	0.85*	0.91*	NS	NS	NS	NS
		Annual Max T (°C)	-	NS	NS	NS	NS	0.90*	NS	0.53*	NS	NS
			m.a.s.l (m)	-	-0.83*	NS	-0.84*	NS	NS	NS	NS	NS
				Precipit. MoG (mm)	-	NS	NS	NS	NS	NS	NS	NS
					MinT MoG (°C)	-	0.94**	NS	NS	NS	NS	NS
						MeanT MoG (°C)	-	0.87*	NS	NS	NS	NS
							MaxT MoG (°C)	-	NS	0.65**	NS	NS
								#T_b (°C)	-		0.66**	NS
									#θ_T (°Ch)	-	NS	0.77***
										#Ψ_b (MPa)	-	NS

9.4 Appendix Chapter 5

*P < 0.05 **P < 0.01 ***P < 0.001	Min T MoG (°C)	Mean T MoG (°C)	Max T MoG (°C)	Mean precipit. (mm)	Annual Min T (°C)	Annual Mean T (°C)	Annual Max T (°C)	m.a.s.l (m)	θ_T (°Ch)	T_b (°C)	θ_H (MPah)	Ψ_b (MPa)
Precipit. MoG (mm)	NS	NS	NS	0.95 **	NS	-0.82 *	-0.88 **	NS	0.82 ***	-0.83 ***	0.61 **	-0.63 **
MinT MoG	-	0.97 ***	0.92 **	NS	NS	NS	NS	NS	NS	NS	NS	NS
	MeanT MoG	-	0.98 ***	NS	NS	NS	NS	NS	NS	NS	NS	NS
		MaxT MoG	-	NS	NS	NS	NS	NS	NS	0.486 *	NS	NS
			Mean precipit.	-	NS	NS	-0.825 *	NS	0.84 ***	-0.83 ***	0.73***	-0.78 ***
				Annual MinT	-	0.97 ***	0.91 **	NS	NS	0.68 ***	NS	NS
					Annual MeanT	-	0.98 ***	NS	NS	0.76 ***	NS	NS
						Annual MaxT	-	NS	-0,52 *	0.80 ***	NS	NS
							m.a.s.l (m)	-	NS	NS	NS	NS
								θ_T (°Ch)	-	-0.76 ***	0.84 ***	-0.72 ***
									T_b (°C)	-	NS	NS
										θ_H (MPah)	-	-0.93 ***

Table A5.1 Table of correlations between physical and physiological seed traits of the *Brassica* genus. Correlation coefficient values (r) of the correlations between environmental factors (mean precipitation of the month of germination (MoG), mean monthly precipitation, annual mean minimum, mean and maximum temperatures (T) of both periods and altitude above sea level (m.a.s.l), seed germination parameters (thermal time, θ_T, hydro time, θ_H, base temperature, T_b and base water potential, Ψ_b) seed mass and the coefficient of variation (CV) of seed mass of seven CWRs of *Brassica*. NS = Not Significant. Asterisks indicating the significance at $P < 0.05$*, $P < 0.01$**, $P < 0.001$***

Figure A5.1 Historical annual mean temperature (minimum in red and maximum in yellow) and mean monthly precipitation (blue bars) in the environment of seed collection site of seven CWRs of *Brassica*. The month enclosed in the orange square symbol represents the dispersal month of the seeds according to the collectors. The green arrows are the predicted month of germination (MoG) when the seeds would be able to germinate according to the assumption of (1) the precipitation was above 15 mm, previous studies suggested this quantity (between 10 and 20) as the minimum rainfall the seeds were able to start the germination (Freas and Kemp, 1983, Gutterman, 1993, Gutterman, 2000a), and (2) the minimum environmental temperature exceeded T_b.

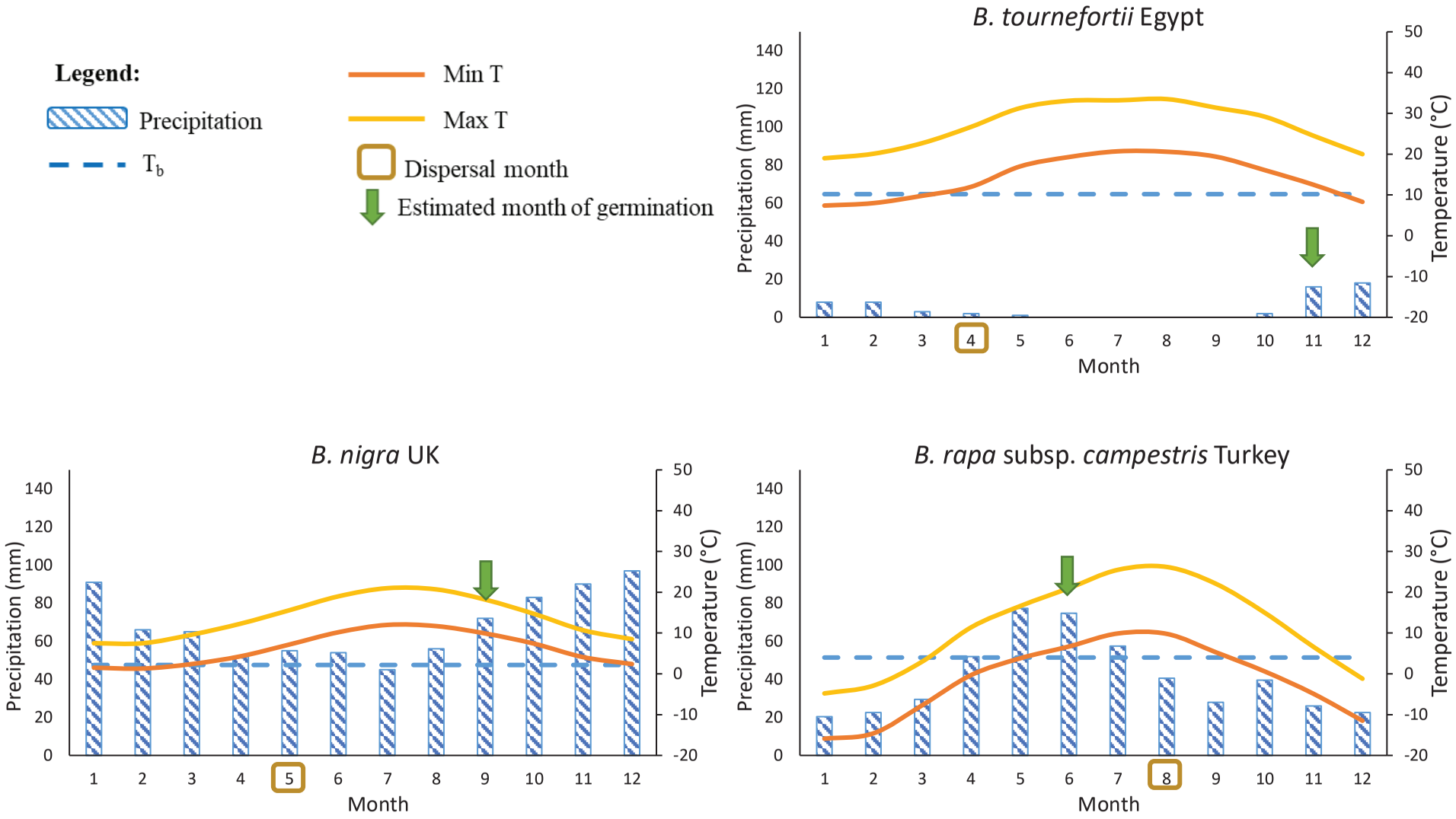


Figure A5.1 Continuation

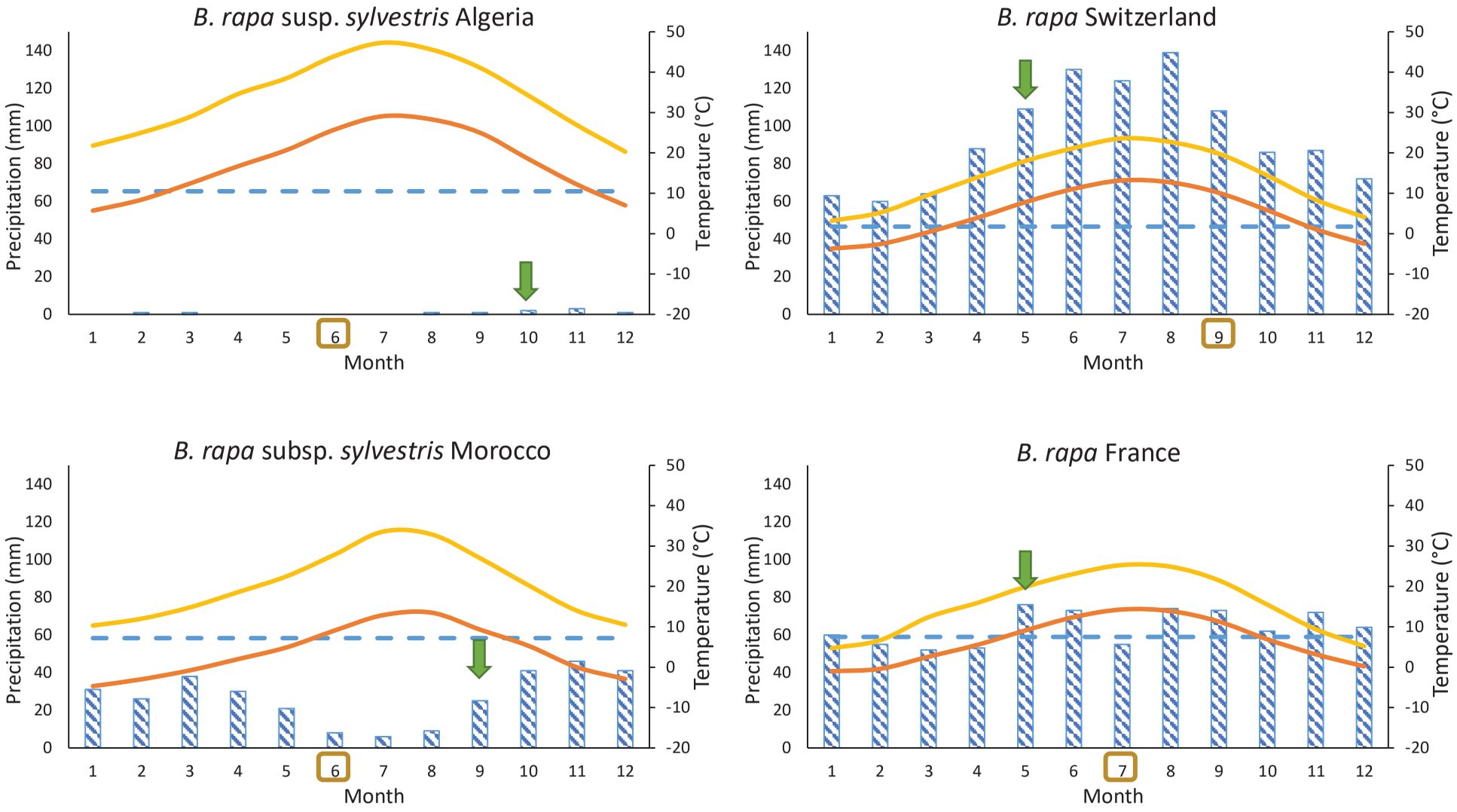


Figure A5.1 Continuation

9.5 Appendix Chapter 6

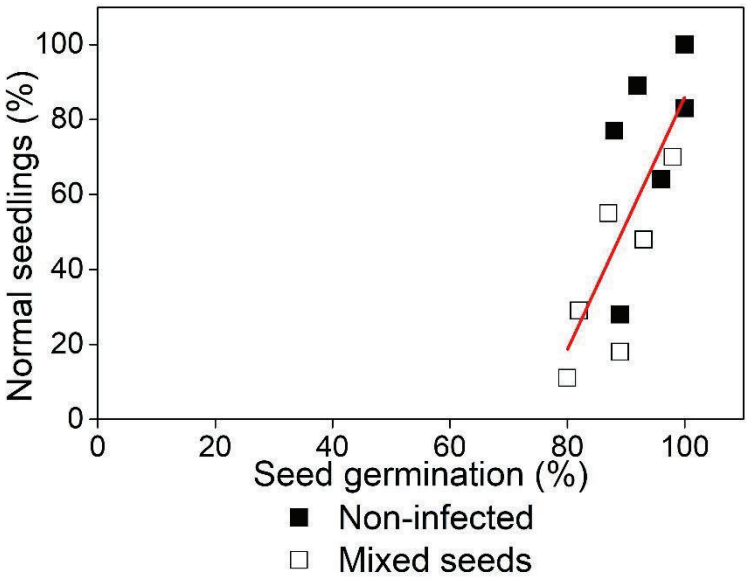


Figure A6.1 Proportional decrease of normal seedlings and seed germination as total percentage for non-infected seeds and mixed seeds of the research crop genotype B of *Helianthus annuus*.

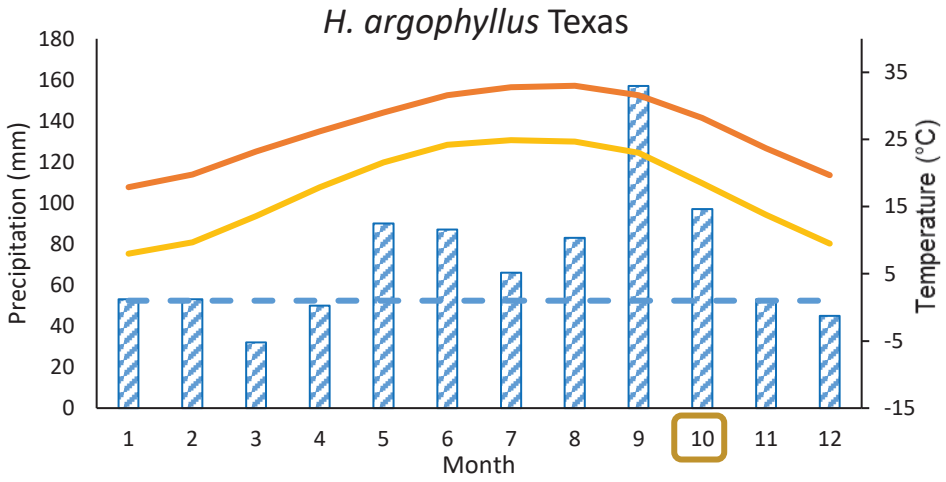


Figure A6.2 Historical annual mean temperature (minimum in red and maximum in yellow) and mean monthly precipitation (blue bars) in the environment of seed collection site of five CWRs of *Helianthus*. The month enclosed in the orange square symbol represents the dispersal month of the seeds according to the collectors. The green arrows are the predicted month of germination (MoG) when the seeds would be able to germinate according to the assumption of (1) the precipitation was above 15 mm, previous studies suggested this quantity (between 10 and 20) as the minimum rainfall the seeds were able to start the germination (Freas and Kemp, 1983, Gutterman, 1993, Gutterman, 2000a), and (2) the minimum environmental temperature exceeded T_b. According to the environmental criteria defined in Chapter 2, section 2.2.2 to predict seed germination, seeds of *H. argophyllus* could germinate in multiple months after seed dispersal.

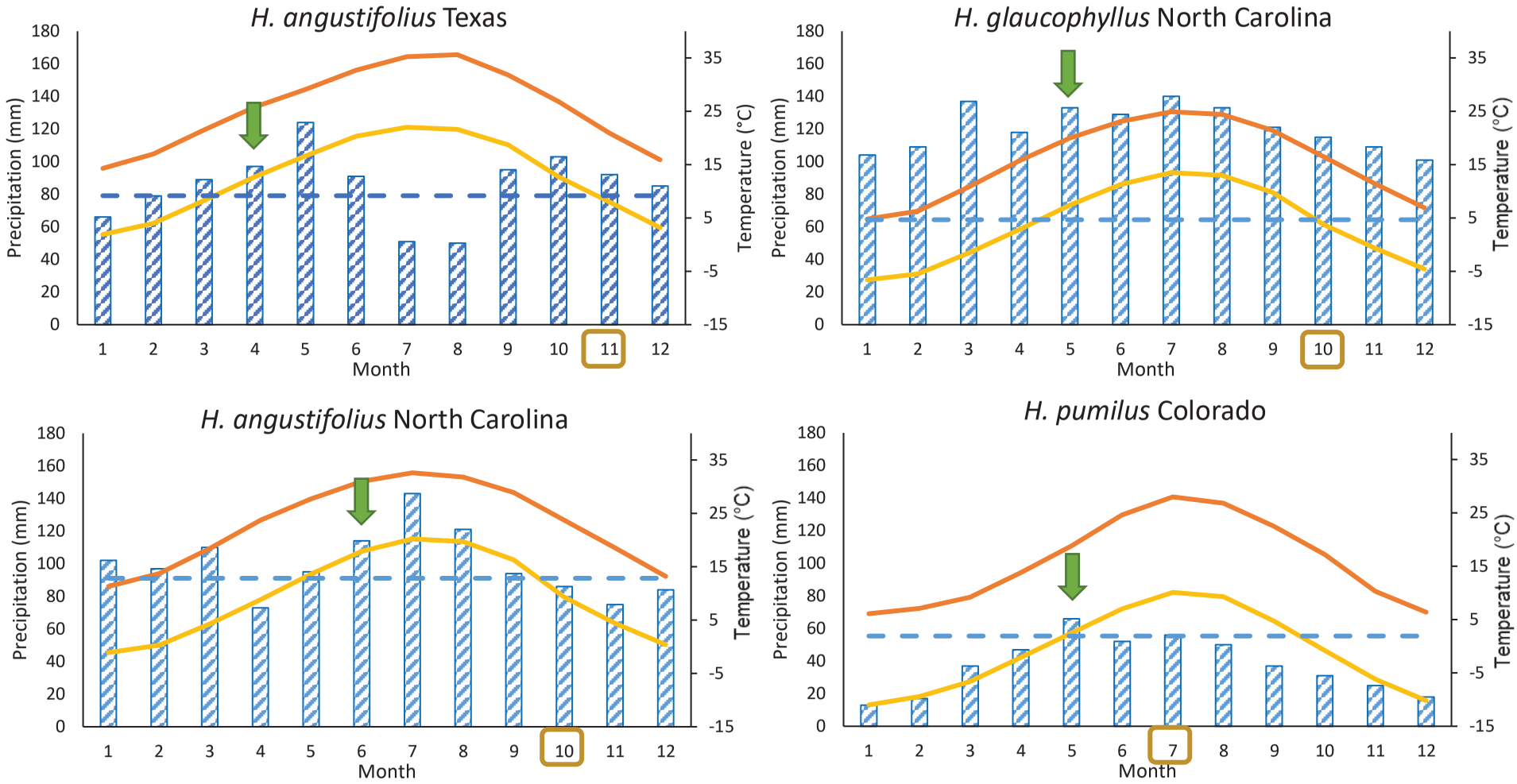


Figure A6.2 Continuation

9.6 Appendix Chapter 7

Table A7.1 Combination of artificial ageing experiments at three temperatures (T °C) and four relative humidities (RH %) to reach a specific moisture content (MC %) in seeds of two *Helianthus annuus* seed lots, stopped (Stop) and normal irrigation (Normal). The viability (V %) was estimated from seed germination at 20 °C. The days of ageing were predicted from the viability equations of Ellis & Roberts, 1980.

T (°C)	RH (%)	MC (%)	Days to age (V.eq. prediction)	Expected V (%)	Days to age (tests)	V % (Stop)	V % (Normal)
40	75	9	0	100	0	97.3	70.7
			6	85	7	80.0	60.0
			10	60	11	55.7	39.2
			11	50	14	29.2	12.7
			15	25	20	10.0	9.5
	60	7.4	0	100	0	88.9	80.7
			13	85	14	89.1	68.4
			22	60	25	66.7	50.6
			24	50	38	76.5	49.8
			31	25	49	50.7	43.4
	45	6.1	0	100	0	97.3	88.9
			32	85	98	87.8	68.0
			51	60	115	66.7	41.5
			57	50	129	69.9	47.3
			74	25	151	52.9	41.5
	30	4.8	0	100	0	97.3	86.7
			86	85	165	82.2	63.8
			139	60	182	67.2	63.7
			156	50	205	72.5	45.9
			201	25	273	60	42.7

Table A7.1 Continuation

T (°C)	RH (%)	MC (%)	Days to age (V.eq. prediction)	Expected V (%)	Days to age (tests)	V % (Stop)	V % (Normal)
30	75	9.6	0	100	0	86.7	80.0
			21	85	21	83.6	60.7
			34	60	40	63.4	39.5
			38	50	52	49.8	25.8
			50	25	70	13.3	1.3
	60	8	0	100	0	88.0	88.0
			74	85	105	86.4	70.2
			76	60	122	74.6	58.7
			86	50	159	63.0	40.9
			110	25	227	45.3	21.3
	45	6.5	0	100	0	91.9	90.7
			106	85	130	92.0	86.3
			170	60	156	85.8	63.5
			191	50	206	86.6	73.3
			246	25	344	52.0	61.3
20	75	10.3	0	100	0	92.1	84.3
			59	85	54	89.3	73.3
			95	60	94	91.0	73.9
			106	50	227	63.5	45.0
			137	25	281	54.7	24.1
	60	8.6	0	100	0	94.7	90.7
			130	85	138	86.7	89.3
			209	60	226	90.7	74.7
			234	50	339	97.3	78.7
			302	25	413	80.0	70.7

Table A7.2 ANOVA results of comparisons between final germination of fresh seeds (Batch 1 and Batch 2) showed in Table 7.1 for each ageing condition (germination percentages were arcsine transformed). Batch 1 DF = 5 and Batch 2 DF = 4, *significantly different $P < 0.05$

Condition (°C - RH) vs fresh seeds	Normal irrigation			Stopped irrigation		
	Mean	F-value	SEM	Mean	F-value	SEM
Batch 1	**0.782**			**0.867**		
40-30	0.783	0.0008	0.05234	0.862	0.0601	0.01884
30-45	0.814	0.4382	0.04802	0.814	5.0808	0.02369
20-75	0.765	0.1136	0.04915	0.824	3.7070	0.02239
20-60	0.814	0.4382	0.04802	0.843	1.5917	0.01891
Batch 2	**0.768**			**0.843**		
40-75	0.658	6.3991	0.04361	0.862	0.8208	0.02147
40-60	0.721	0.5784	0.06237	0.774	9.4286*	0.02253
40-45	0.783	0.0698	0.05549	0.862	2.1269	0.01347
30-75	0.732	0.4323	0.05553	0.782	1.2761	0.05369
30-60	0.794	0.3665	0.04222	0.794	6.1266	0.01978

Table A7.3 Comparison of the seed moisture content (MC %) based on wet weight between the normal and stopped irrigation treatments of *Helianthus annuus* at the initial time interval (t_1) after equilibrating the seeds at specific relative humidity (eRH) at 20 °C (see Table 7.1) and, separately at the last time interval (t_5) of the ageing experiment. The moisture content values are the means (± SD) of 10 seeds as individual replicates for each ageing condition. Different letters denote significant differences ($P < 0.05$) between normal and stopped irrigation for each ageing condition and each time interval (t_1 and t_5).

		Moisture content (%)	
		After eRH t_1	**End of ageing t_5**
⸸40°C – 75 % RH	Normal	8.179 ± 0.18 a	7.701 ± 0.46 a
	Stopped	7.620 ± 0.14 **b**	7.425 ± 0.40 a
⸸40°C – 60 % RH	Normal	6.249 ± 0.19 a	5.993 ± 0.35 a
	Stopped	5.760 ± 0.08 **b**	5.434 ± 0.34 **b**
⸸40°C – 45 % RH	Normal	5.188 ± 0.11 a	4.702 ± 0.25 a
	Stopped	4.776 ± 0.09 **b**	4.445 ± 0.22 a
40°C – 30 % RH	Normal	4.646 ± 0.39 a	3.980 ± 0.20 a
	Stopped	4.368 ± 0.30 a	3.813 ± 0.16 a
⸸30°C – 75 % RH	Normal	8.564 ± 0.14 a	8.171 ± 0.81 a
	Stopped	7.620 ± 0.14 **b**	7.192 ± 0.36 **b**
⸸30°C – 60 % RH	Normal	7.125 ± 0.15 a	6.233 ± 0.27 a
	Stopped	6.677 ± 0.24 a	5.850 ± 0.30 **b**
30°C – 45 % RH	Normal	5.802 ± 0.32 a	4.984 ± 0.39 a
	Stopped	5.431 ± 0.44 a	4.975 ± 0.24 a
20°C – 75 % RH	Normal	8.628 ± 0.18 a	8.357 ± 0.51 a
	Stopped	8.335 ± 0.49 a	7.933 ± 0.71 a
20°C – 60 % RH	Normal	7.129 ± 0.59 a	6.771 ± 0.52 a
	Stopped	7.078 ± 0.41 a	6.480 ± 0.51 a

⸸ Ageing conditions performed on Batch 2, stored for longer time at 70 % RH at room temperature

Table A7.4 Linear equations from the regression lines showed in Figure 7.2 and 7.3 of seed germination expressed as probit units (y) and constant ageing conditions of three temperatures (T, °C) and relative humidities (RH, %). The coefficient of correlation (r) shows a good relationship between the probit value of the viability (y) and the ageing period in days (x).

T (°C) – RH (%)	Linear equation	r	P-value
Stop 40-75	y = -0.16090 x + 6.88	-0.71	<0.001
Normal 40-75	y = -0.13445 x + 6.13	-0.72	<0.001
Stop 40-60	y = -0.03878 x + 6.88	-0.77	<0.001
Normal 40-60	y = -0.03083 x + 6.13	-0.77	<0.001
Stop 40-45	y = -0.01105x + 6.88	-0.83	<0.001
Normal 40-45	y = -0.00935 x + 6.13	-0.83	<0.001
Stop 40-30	y = -0.00630 x + 6.88	-0.83	<0.001
Normal 40-30	y = -0.00496 x + 6.13	-0.83	<0.001
Stop 30-75	y = -0.04035 x + 6.88	-0.72	<0.001
Normal 30-75	y = -0.03909 x + 6.13	-0.70	<0.001
Stop 30-60	y = -0.00924 x + 6.88	-0.79	<0.001
Normal 30-60	y = -0.00778 x + 6.13	-0.78	<0.001
Stop 30-45	y = -0.00441 x + 6.88	-0.78	<0.001
Normal 30-45	y = -0.00302 x + 6.13	-0.79	<0.001
Stop 20-75	y = -0.00653 x + 6.88	-0.71	<0.001
Normal 20-75	y = -0.00620 x + 6.13	-0.70	<0.001
Stop 20-60[+]	y = -0.00184 x + 6.88	-0.82	<0.001
Normal 20-60[+]	y = -0.00123 x + 6.13	-0.82	<0.001

[+] DF = 3, in general the degrees of freedom are 4 or more

Table A7.5 Linear equations from the regression lines shown in Figure 7.4 and 7.5 of p50 (days a seed lot needs to lose 50 % of the viability) at constant temperature (T, °C) at different relative humidities (RH, %, Figure 7.4) and vice versa (Figure 7.5). The coefficient of correlation (r) shows a good relationship between the p50 (y) and the RH (at constant temperature) or temperature (at constant RH, x).

Constant T (°C)	Linear equation	r	P-value
Stop 40	y = -6.42991 x + 471.08002	-0.98	<0.05
Normal 40	y = -4.98200 x + 359.57932	-0.97	<0.05
Stop 30[+]	y = -14.69915 x + 1135.2038	-0.99	NS
Normal 30[+]	y = -10.67560 x + 812.99045	-0.99	NS
Constant RH (%)	Logarithmic scale		
Stop 75[+]	y = -0.07019 x + 3.83712	-0.99	NS
Normal 75[+]	y = -0.07086 x + 3.63592	-0.99	NS
Stop 60[+]	y = -0.07779 x + 4.79997	-0.99	NS
Normal 60[+]	y = -0.06431 x + 4.12272	-0.99	<0.05

[+] DF = 1

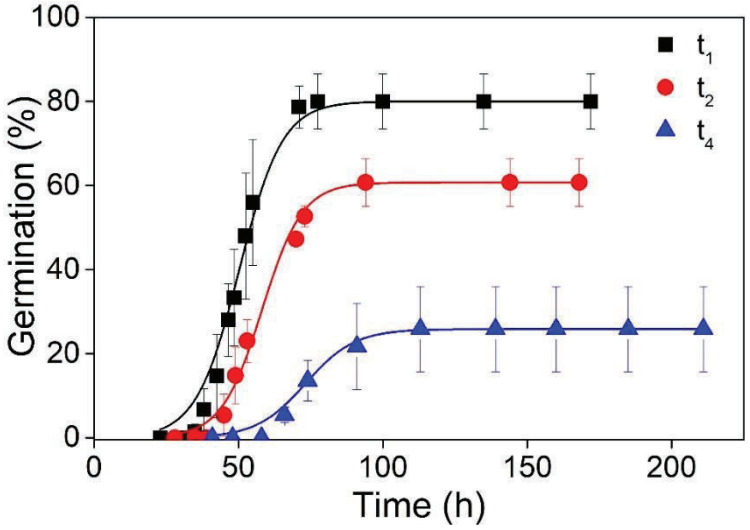


Figure A7.1 Example of cumulative germination during ageing of the three time intervals, t_1 t_2 and t_4. The seeds are from the normal irrigation treatment at 30 °C– 75 % RH germinated at 20 °C.

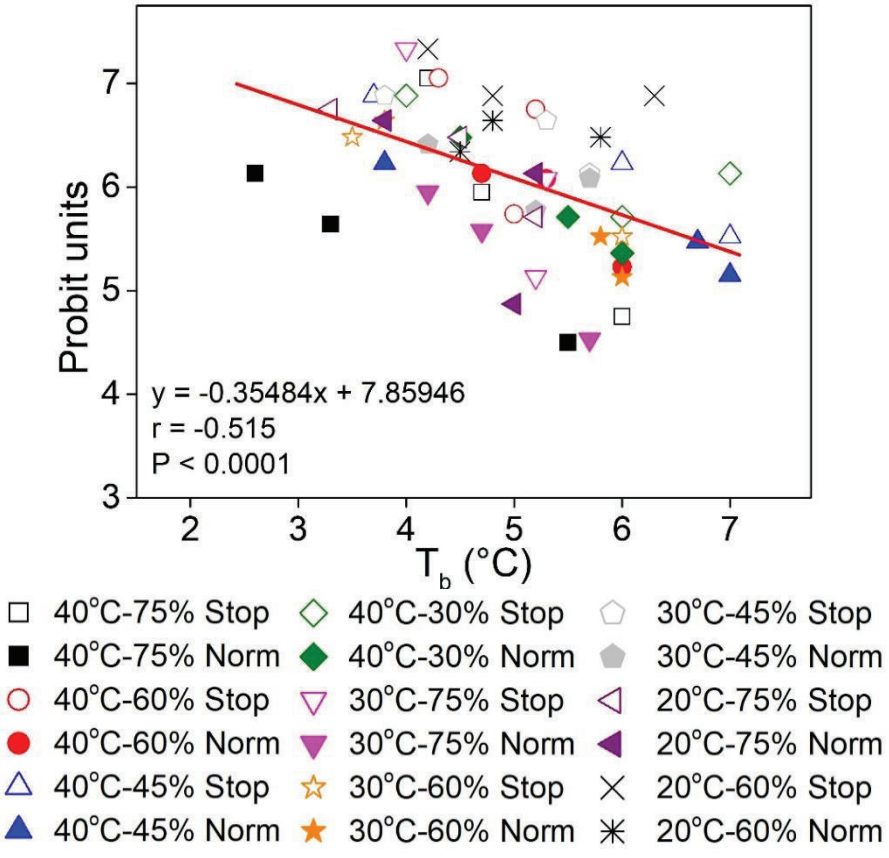


Figure A7.2 Correlation between the viability in probit units and base temperature, T_b, for each ageing condition for the time intervals t_1, t_2 and t_4 on *Helianthus annuus* seeds from stopped irrigation (Stop, open symbols) and normal irrigation (Norm, solid symbols) treatments analysed together.

Table A7.6 Seed germination parameters of *H. annuus* (normal and stopped irrigation) calculated during the ageing experiment (at constant temperature and relative humidity, RH) at three time intervals t_1 (after seed equilibration), t_2 and t_4 (ageing days/viability percentage, V %, of the seed lot after days of ageing). The data was calculated using the repeated probit analysis and are the mean of three replicates for base temperature (T_b), thermal time in the sub-optimal range of temperatures (θ_T), base water potential (Ψ_b) and hydro time (θ_H). Different letters denote significant differences ($P < 0.05$) between the three time intervals. Shaded cells highlight the main significant changes between time intervals.

Table A7.7 Percentage of normal seedlings as a proportion of the number of germinated seeds for each germination condition (i.e. three constant temperatures and water potentials) and time intervals. Percentages were arcsine transformed before statistical analysis by Tukey test to compare the time intervals t_1 (after seed equilibration), t_2 and t_4. The comparisons were performed for each ageing condition and each germination condition. The seeds subjected at -0.3MPa and -0.5MPa were imbibed at 20°C.

Table A7.6 Continuation	T_b	θ_T	Ψ_b	θ_H
Time interval (days/V %)	**40 °C – 75 % RH Normal irrigation**			
t_1 (0 / 70.7)	2.6 ± 1.1 a	849.2 ± 130.7 a	-1.073 ± 0.290 a	63.0 ± 23.8 b
t_2 (7 / 60.0)	3.3 ± 1.7 a	1210.7 ± 130.6 a	-1.143 ± 0.226 a	81.7 ± 17.0 ab
t_4 (14 / 12.7)	5.5 ± 0.7 a	1295.3 ± 96.6 a	-1.405 ± 0.0 a	130.0 ± 0.0 a
	40 °C – 60 % RH Normal irrigation			
t_1 (0 / 80.7)	4.7 ± 0.5 a	808.0 ± 60.3 a	-1.125 ± 0.056 a	63.3 ± 4.7 a
t_2 (14 / 68.4)	5.3 ± 0.2 a	846.4 ± 37.7 a	-0.945 ± 0.093a	55.0 ± 7.1 a
t_4 (38 / 49.8)	6.0 ± 0.7 a	1000.9 ± 103.8 a	-1.200 ± 0.262 a	93.3 ± 26.6 a
	40 °C – 45 % RH Normal irrigation			
t_1 (0 / 88.9)	3.8 ± 0.5 b	848.8 ± 57.7 a	-1.038 ± 0.099 a	55.0 ± 7.1 a
t_2 (98 / 68.0)	6.7 ± 0.2 a	811.9 ± 22.1 a	-0.901 ± 0.177 a	56.7 ± 14.3 a
t_4 (129 / 47.3)	7.0 ± 1.1 a	876.5 ± 98.0 a	-0.920 ± 0.254 a	66.7 ± 21.0 a
	40 °C – 30 % RH Normal irrigation			
t_1 (0 / 86.7)	4.5 ± 0.0 a	772.1 ± 11.3 a	-0.801 ± 0.062 a	40.0 ± 4.1 b
t_2 (165 / 63.8)	5.5 ± 0.8 a	850.7 ± 90.8 a	-0.995 ± 0.048 ab	61.7 ± 2.4 ab
t_4 (205/ 45.9)	6.0 ± 0.4 a	836.8 ± 73.2 a	-1.233 ± 0.206 b	83.3 ± 18.9 a

Table A7.6 Continuation	T_b	θ_T	Ψ_b	θ_H
Time interval (days/V %)	**40 °C – 75 % RH Stopped irrigation**			
t_1 (0 / 97.3)	4.2 ± 0.2 b	828.5 ± 22.0 b	-1.091 ± 0.100 a	58.0 ± 7.3 b
t_2 (7 / 80.0)	4.7 ± 0.5 ab	909.0 ± 65.4 ab	-1.032 ± 0.070 a	61.7 ± 6.2 b
t_4 (14 / 29.2)	6.0 ± 0.5 a	1075.6 ± 4.0 a	-1.463 ± 0.120 b	130.0 ± 16.3 a
	40 °C – 60 % RH Stopped irrigation			
t_1 (0 / 88.9)	4.3 ± 0.2 a	806.3 ± 30.0 a	-1.191 ± 0.885 a	65.0 ± 7.1 a
t_2 (14 / 89.1)	5.2 ± 0.5 a	837.3 ± 41.8 a	-1.050 ± 0.127 a	63.3 ± 9.4 a
t_4 (38 / 76.5)	5.0 ± 0.4 a	1101 ± 40.1 b	-1.497 ± 0.305 a	101.7 ± 20.9 b
	40 °C – 45 % RH Stopped irrigation			
t_1 (0 / 97.3)	3.7 ± 0.2 c	822.1 ± 20.7 a	-1.355 ± 0.153 a	71.7 ± 10.3 a
t_2 (98 / 87.8)	6.0 ± 0.0 b	853.9 ± 10.1 a	-1.168 ± 0.203 a	75.0 ± 14.1 a
t_4 (129 / 69.9)	7.0 ± 0.4 a	802.1 ± 38.7 a	-0.939 ± 0.061 a	63.3 ± 4.7 a
	40 °C – 30 % RH Stopped irrigation			
t_1 (0 / 97.3)	4.0 ± 0.7 c	741.0 ± 50.1 a	-0.950 ± 0.097 a	45.0 ± 7.1 b
t_2 (165 / 82.2)	7.0 ± 0.0 a	695.9 ± 16.4 a	-1.050 ± 0.142 ab	60.0 ± 8.2 ab
t_4 (205/ 72.5)	6.0 ± 0.4 b	798.0 ± 46.8 a	-1.274 ± 0.053 b	76.7 ± 4.7 a

Table A7.6 Continuation	T_b	θ_T	Ψ_b	θ_H
Time interval (days/V %)	**30 °C – 75 % RH Normal irrigation**			
t_1 (0 / 80.0)	4.2 ± 0.2 a	832.2 ± 25.1 b	-0.995 ± 0.246 a	51.7 ± 16.5 a
t_2 (21 / 60.7)	4.7 ± 0.2 a	951.7 ± 17.2 ab	-1.119 ± 0.118 a	71.7 ± 9.4 a
t_4 (52 / 25.8)	5.7 ± 0.9 a	1092.0 ± 111.0 a	-1.020 ± 0.375 a	82.5 ± 37.5 a
	30 °C – 60 % RH Normal irrigation			
t_1 (0 / 88.0)	3.8 ± 0.5 b	863.8 ± 48.4 a	-0.896 ± 0.022 a	46.7 ± 2.4 b
t_2 (105 / 70.2)	5.8 ± 0.2 a	854.8 ± 22.3 a	-0.891 ± 0.040 a	55..0 ± 4.1 b
t_4 (159 / 40.9)	6.0 ± 0.0 a	986.5 ± 44.2 a	-1.373 ± 0.191 b	97.5 ± 12.5 a
	30 °C – 45 % RH Normal irrigation			
t_1 (0 / 90.7)	4.2 ± 0.2 b	825.5 ± 23.7 a	-0.774 ± 0.098 a	38.3 ± 4.7 b
t_2 (130 / 86.3)	5.7 ± 0.2 a	812.0 ± 17.9 a	-0.915 ± 0.068 a	51.7 ± 2.4 a
t_4 (206 / 73.3)	5.2 ± 0.6 a	895.0 ± 119.2 a	-0.966 ± 0.036 a	55.0 ± 5.0 a

Table A7.6 Continuation	T_b	θ_T	Ψ_b	θ_H
Time interval (days/V %)	**30 °C – 75 % RH Stopped irrigation**			
t_1 (0 / 86.7)	4.0 ± 0.4 b	799.4 ± 42.9 b	-1.130 ± 0.082 ab	58.3 ± 6.2 b
t_2 (21 / 83.6)	5.3 ± 0.2 a	876.9 ± 46.2 b	-0.952 ± 0.096 a	56.7 ± 6.2 b
t_4 (52 / 49.8)	5.2 ± 0.6 a	1182.0 ± 62.2 a	-1.236 ± 0.100 b	93.3 ± 9.4 a
	30 °C – 60 % RH Stopped irrigation			
t_1 (0 / 88.0)	3.5 ± 0.7 b	828.8 ± 43.8 a	-1.170 ± 0.208 a	61.7 ± 13.1 a
t_2 (105 / 86.4)	5.3 ± 0.2 a	901.8 ± 39.9 a	-0.919 ± 0.090 a	58.3 ± 6.2 a
t_4 (159 / 63)	6.0 ± 0.4 a	864.9 ± 57.4 a	-0.994 ± 0.054 a	63.3 ± 4.7 a
	30 °C – 45 % RH Stopped irrigation			
t_1 (0 / 91.9)	3.8 ± 0.5 b	777.4 ± 40.0 a	-0.986 ± 0.069 a	48.3 ± 6.2 b
t_2 (130 / 92)	5.3 ± 0.2 a	793.4 ± 22.9 a	-1.088 ± 0.117 a	60.0 ± 8.2 a
t_4 (206 / 86.6)	5.7 ± 0.2 a	792.1 ± 23.3 a	-1.047 ± 0.066 a	58.3 ± 2.4 a

Table A7.6 Continuation	T_b	θ_T	Ψ_b	θ_H
Time interval (days/V %)		**20 °C – 75 % RH Normal irrigation**		
t_1 (0 / 84.3)	3.8 ± 0.2 a	843.5 ± 10.9 a	-0.955 ± 0.094 a	50.0 ± 5.0 a
t_2 (54 / 73.3)	5.2 ± 0.5 a	816.2 ± 39.7 a	-0.942 ± 0.132 a	58.3 ± 9.4 a
t_4 (227 / 45.0)	5.0 ± 1.1 a	971.2 ± 127.7 a	-1.260 ± 0.181 a	78.3 ± 12.5 a
		20 °C – 60 % RH Normal irrigation		
t_1 (0 / 90.7)	4.5 ± 0.4 b	788.9 ± 34.3 a	-0.905 ± 0.096 a	48.3 ± 4.7 a
t_2 (138 / 89.3)	4.8 ± 0.5 ab	849.9 ± 78.8 a	-0.996 ± 0.118 a	53.3 ± 8.5 a
t_4 (339 / 78.7)	5.8 ± 0.2 a	779.8 ± 33.0 a	-	-
		20 °C – 75 % RH Stopped irrigation		
t_1 (0 / 92.1)	3.3 ± 0.2 b	800.5 ± 7.7 ab	-0.940 ± 0.105 a	45.0 ± 8.2 b
t_2 (54 / 89.3)	4.5 ± 0.8 ab	781.6 ± 52.7 b	-0.945 ± 0.026 a	51.7 ± 2.4 b
t_4 (227 / 63.5)	5.2 ± 0.5 a	948.9 ± 67.0 a	-1.061 ± 0.011 a	68.3 ± 2.4 a
		20 °C – 60 % RH Stopped irrigation		
t_1 (0 / 94.7)	4.2 ± 0.9 b	739.3 ± 64.7 a	-0.973 ± 0.084 a	48.3 ± 4.7 a
t_2 (138 / 86.7)	4.8 ± 0.6 ab	773. 2 ± 74.3 a	-1.192 ± 0.229 ab	60.0 ± 16.3 ab
t_4 (339 / 97.3)	6.3 ± 0.2 a	690.7 ± 9.7 a	-1.720 ± 0.089 b	85.0 ± 5.0 b

Table A7.7 Continuation

Time interval (days/V %)	Proportion of Normal seedling (%)				
	10 °C	**20 °C**	**25 °C**	**-0.3 MPa**	**-0.5 MPa**
	40 °C - 75 RH Normal irrigation				
t_1 (0 / 70.7)	92.7 ± 5.3 a	67.8 ± 7.8 a	47.8 ± 7.5 a	70.1 ± 16.8 a	49.3 ± 6.2 b
t_2 (7 / 60.0)	61.7 ± 13.1 ab	71.0 ± 2.8 a	56.3 ± 11.6 a	71.8 ± 9.6 a	71.4 ± 11.7 a
t_4 (14 / 12.7)	53.0 ± 14.0 b	22.2 ± 15.7 b	45.6 ± 20.9 a	41.9 ± 15.6 a	39.2 ± 1.2 b
	40 °C - 60 RH Normal irrigation				
t_1 (0 / 80.7)	81.5 ± 10.5 a	76.1 ± 4.8 b	0.0 ± 0.0 b	63.6 ± 27.5 a	66.3 ± 24.1 a
t_2 (14 / 68.4)	20.0 ± 4.4 b	100.0 ± 0.0 a	35.3 ± 12.0 a	46.5 ± 9.7 a	50.0 ± 9.4 a
t_4 (38 / 49.8)	50.1 ± 5.2 c	48.6 ± 10.0 c	57.5 ± 11.3 a	50.7 ± 19.6 a	26.3 ± 20.1 a
	40 °C - 45 RH Normal irrigation				
t_1 (0 / 88.9)	90.9 ± 6.4 a	65.2 ± 15.0 a	3.0 ± 4.3 b	57.8 ± 16.1 a	65.7 ± 11.2 a
t_2 (98 / 68.0)	48.5 ± 5.1 b	52.8 ± 2.3 a	32.8 ± 6.1 a	61.0 ± 8.1 a	45.7 ± 10.2 a
t_4 (129 / 47.3)	47.6 ± 14.4 b	66.8 ± 8.1 a	42.6 ± 8.0 a	46.9 ± 2.3 a	40.7 ± 16.6 a
	40 °C - 30 RH Normal irrigation				
t_1 (0 / 86.7)	81.3 ± 4.4 a	84.7 ± 1.6 a	57.5 ± 18.2 a	80.1 ± 11.3 a	62.7 ± 19.8 a
t_2 (165 / 63.8)	63.1 ± 21.1 a	55.3 ± 12.6 b	50.4 ± 4.2 a	64.6 ± 14.9 a	51.0 ± 12.4 a
t_4 (205/ 45.9)	57.5 ± 11.6 a	69.8 ± 2.5 ab	56.0 ±7.2 a	63.0 ± 5.6 a	56.4 ± 10.2 a

Table A7.7 Continuation

Time interval (days/V %)	Proportion of Normal seedling (%)				
	10 °C	**20 °C**	**25 °C**	**-0.3 MPa**	**-0.5 MPa**
	40 °C - 75 RH Stopped irrigation				
t_1 (0 / 97.3)	94.6 ± 1.9 a	58.8 ± 3.7 ab	53.3 ± 13.8 a	81.8 ± 13.2 a	65.5 ± 4.2 a
t_2 (7 / 80.0)	82.4 ± 4.1 b	68.5 ± 8.2 a	55.1 ± 8.6 a	71.0 ± 11.2 a	76.6 ± 8.8 a
t_4 (14 / 29.2)	47.8 ± 11.0 c	47.6 ± 6.7 b	32.7 ± 4.2 b	32.5 ± 24.7 a	64.8 ± 11.4 a
	40 °C - 60 RH Stopped irrigation				
t_1 (0 / 88.9)	83.7 ± 11.9 a	82.4 ± 10.4 a	0.0 ± 0.0 b	79.1 ± 12.4 a	77.5 ± 8.0 a
t_2 (14 / 89.1)	20.0 ± 2.2 b	50.8 ± 14.3 b	56.8 ± 11.6 a	64.8 ± 3.8 a	52.1 ± 7.1 b
t_4 (38 / 76.5)	34.3 ± 21.3 b	59.3 ± 4.9 ab	53.4 ± 14.2 a	56.1 ± 16.2 a	79.4 ± 3.4 a
	40 °C - 45 RH Stopped irrigation				
t_1 (0 / 97.3)	82.6 ± 7.0 a	68.1 ± 5.2 a	1.5 ± 2.1 b	75.7 ± 8.6 a	85.7 ± 8.0 a
t_2 (98 / 87.8)	64.6 ± 13.1 a	61.6 ± 3.9 a	28.5 ± 9.1 a	49.5 ± 12.9 b	71.9 ± 4.7 a
t_4 (129 / 69.9)	29.6 ± 10.2 b	61.3 ± 11.9 a	46.3 ± 5.2 a	77.9 ± 4.1 a	57.8 ± 26.2 a
	40 °C - 30 RH Stopped irrigation				
t_1 (0 / 97.3)	81.8 ± 5.8 a	75.3 ± 9.1 a	57.2 ± 8.8 a	73.9 ± 1.6 a	81.2 ± 16.0 a
t_2 (165 / 82.2)	72.6 ± 12.4 ab	47.9 ± 10.6 b	56.9 ± 4.9 a	59.6 ± 11.0 a	74.9 ± 13.1 a
t_4 (205/ 72.5)	54.8 ± 7.3 b	59.8 ± 5.7 ab	60.7 ± 18.2 a	42.5 ± 16.4 a	63.2 ± 16.0 a

Table A7.7 Continuation

Time interval (days/V %)	Proportion of Normal seedling (%)				
	10 °C	**20 °C**	**25 °C**	**-0.3 MPa**	**-0.5 MPa**
	30 °C - 75 RH Normal irrigation				
t_1 (0 / 80.0)	98.4 ± 2.2 a	71.8 ± 5.3 a	45.2 ± 8.5 a	60.8 ± 9.8 a	83.0 ± 7.4 a
t_2 (21 / 60.7)	69.3 ± 10.9 a	56.2 ± 6.5 a	47.5 ± 8.3 a	61.0 ± 3.1 a	67.5 ± 8.9 a
t_4 (52 / 25.8)	36.1 ± 10.4 b	48.3 ± 22.5 a	25.4 ± 8.1 a	50.9 ± 7.0 a	68.2 ± 2.2 a
	30 °C - 60 RH Normal irrigation				
t_1 (0 / 88.0)	80.2 ± 5.3 a	59.1 ± 3.7 a	19.2 ± 8.0 b	68.1 ± 5.2 a	65.8 ± 2.1 a
t_2 (105 / 70.2)	56.7 ± 4.7 ab	53.1 ± 10.9 a	28.0 ± 7.7 b	64.5 ± 21.9 a	39.9 ± 7.2 b
t_4 (159 / 40.9)	32.2 ± 13.4 b	33.2 ± 19.0 a	62.3 ± 6.7 a	43.5 ± 2.6 a	57.4 ± 6.9 ab
	30 °C - 45 RH Normal irrigation				
t_1 (0 / 90.7)	75.3 ± 0.5 a	69.2 ± 9.2 a	44.4 ± 8.1 a	54.8 ± 7.8 a	79.1 ± 14.9 a
t_2 (130 / 86.3)	83.6 ± 8.3 a	54.8 ± 0.6 a	52.7 ± 7.5 a	80.6 ± 23.4 a	70.4 ± 9.4 a
t_4 (206 / 73.3)	73.1 ± 1.3 a	66.3 ± 10.5 a	44.4 ± 4.5 a	50.2 ± 13.6 a	75.3 ± 5.9 a

Table A7.7 Continuation

Time interval (days/V %)	Proportion of Normal seedling (%)				
	10 °C	**20 °C**	**25 °C**	**-0.3 MPa**	**-0.5 MPa**
	30 °C - 75 RH Stopped irrigation				
t_1 (0 / 86.7)	88.8 ± 8.9 a	64.2 ± 11.9 a	54.2 ± 6.2 a	74.3 ± 14.5 a	81.4 ± 4.3 a
t_2 (21 / 83.6)	76.8 ± 5.2 a	68.8 ± 2.8 a	54.5 ± 3.3 a	65.3 ± 13.4 a	66.4 ± 11.9 ab
t_4 (52 / 49.8)	38.9 ± 10.4 b	49.6 ± 3.2 a	26.7 ± 4.7 b	56.0 ± 11.0 a	55.3 ± 1.6 b
	30 °C - 60 RH Stopped irrigation				
t_1 (0 / 88.0)	82.7 ± 10.2 a	42.7 ± 12.1 a	48.7 ± 11.7 a	77.4 ± 8.5 a	74.0 ± 8.6 a
t_2 (105 / 86.4)	77.3 ± 3.8 a	49.8 ± 3.9 a	54.3 ± 4.2 a	48.6 ± 5.6 b	58.5 ± 9.5 a
t_4 (159 / 63)	64.4 ± 16.4 a	56.9 ± 10.9 a	69.1 ± 7.6 a	59.6 ± 9.9 ab	47.9 ± 12.4 a
	30 °C - 45 RH Stopped irrigation				
t_1 (0 / 91.9)	78.2 ± 4.5 a	76.3 ± 12.2 a	52.7 ± 5.8 a	73.7 ± 14.3 a	72.9 ± 10.2 a
t_2 (130 / 92)	83.1 ± 3.4 a	55.2 ± 17.1 a	63.6 ± 15.8 a	58.0 ± 13.5 a	74.1 ± 7.4 a
t_4 (206 / 86.6)	85.0 ± 2.0 a	63.4 ± 5.5 a	42.3 ± 9.0 a	65.9 ± 5.7 a	66.5 ± 19.3 a

Table A7.7 Continuation

Time interval (days/V %)	Proportion of Normal seedling (%)				
	10 ℃	**20 ℃**	**25 ℃**	**-0.3 MPa**	**-0.5 MPa**
	20 ℃ - 75 RH Normal irrigation				
t_1 (0 / 84.3)	97.2 ± 3.9 a	40.7 ± 31.3 a	46.2 ± 12.3 a	59.9 ± 7.2 ab	79.9 ± 6.0 a
t_2 (54 / 73.3)	90.8 ± 0.4 a	75.7 ± 8.3 a	23.7 ± 16.8 a	29.8 ± 12.3 b	66.2 ± 12.0 a
t_4 (227 / 45.0)	49.1 ± 17.6 b	51.1 ± 15.0 a	22.2 ± 7.9 a	69.0 ± 10.1 a	71.8 ± 1.3 a
	20 ℃ - 60 RH Normal irrigation				
t_1 (0 / 90.7)	96.9 ± 2.2 a	63.2 ± 2.9 a	53.2 ± 6.3 a	52.2 ± 17.8 a	67.9 ± 7.1 a
t_2 (138 / 89.3)	78.5 ± 13.0 a	65.4 ± 10.2 a	38.5 ± 8.8 a	55.1 ± 18.7 a	75.5 ± 20.9 a
t_4 (339 / 78.7)	98.5 ± 2.1 a	64.7 ± 5.4 a	28.8 ± 14.5 a	61.8 ± 19.2 a	47.5 ± 4.6 a
	20 ℃ - 75 RH Stopped irrigation				
t_1 (0 / 92.1)	94.6 ± 5.0 a	66.9 ± 5.6 ab	44.2 ± 6.7 a	80.2 ± 13.4 a	83.5 ± 4.8 a
t_2 (54 / 89.3)	90.9 ± 7.0 a	80.7 ± 7.3 a	0.0 ± 0.0 b	58.9 ± 9.6 a	60.4 ± 9.7 a
t_4 (227 / 63.5)	44.1 ± 4.2 b	57.6 ± 6.4 b	28.6 ± 11.7 a	78.1 ± 11.2 a	66.0 ± 9.0 a
	20 ℃ - 60 RH Stopped irrigation				
t_1 (0 / 94.7)	91.7 ± 11.8 a	64.6 ± 16.2 a	51.3 ± 6.6 a	66.2 ± 10.2 a	81.5 ± 15.4 a
t_2 (138 / 86.7)	76.6 ± 5.3 a	75.2 ± 2.8 a	39.8 ± 11.9 a	51.5 ± 15.5 a	56.8 ± 6.2 a
t_4 (339 / 97.3)	92.5 ± 2.0 a	48.0 ± 10.6 a	11.3 ± 2.1 b	74.9 ± 2.9 a	62.7 ± 2.8 a

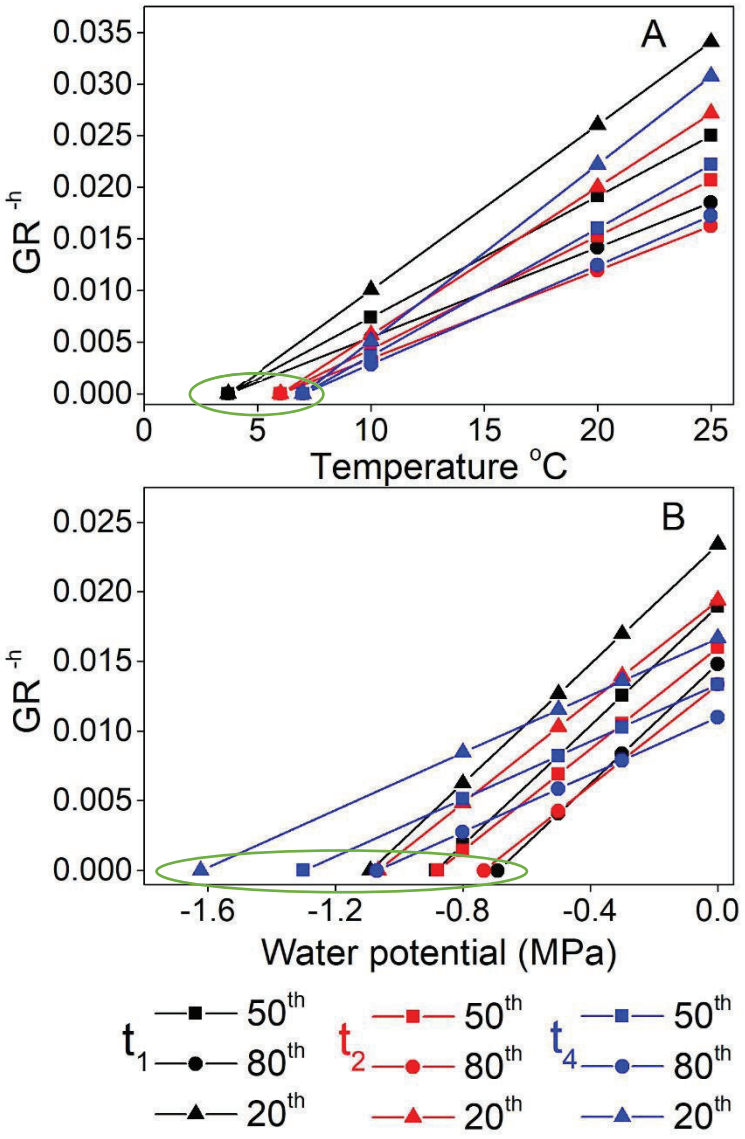


Figure A7.3 Example of the regression lines obtained from the repeated probit analysis values for temperatures (A) and water potentials (B) for the three ageing intervals, t_1 (black) t_2 (red) and t_4 (blue) and three percentiles each.

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